Genomic conflict and disparity within basidiomycete mycelia

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GENOMIC CONFLICT AND DISPARITY WITHIN BASIDIOMYCETE MYCELIA

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ABSTRACT

Mating in the basidiomycetous fungi brings together populations of nuclei and organelles that are capable of forming diverse and unstable relationships. Genomic disparities within a cell, either *genetic* (as a result of non-self fusions *per se*), or *epigenetic* (as a result of fusions between cells in alternative developmental states) engenders the possibility for conflict, coercion and / or confusion at the cellular level. According to circumstance this may lead to instabilities at subsequent developmental, population biological and evolutionary scales of organization.

These issues have been examined in the context of naturally-occurring and laboratory-synthesized heterokaryons of two fungi that produce mycelia with multinucleate hyphal compartments, *Heterobasidion annosum* and *Stereum hirsutum*. Using a combination of fluorescence light microscopy, molecular DNA fingerprinting and laboratory-based pairing experiments, nuclear and mitochondrial genomes have been followed through a variety of sib-related, non-sib-related sympatric and non-sib-related allopatric heterokaryotic associations.

Patterns of nuclear and mitochondrial exchange revealed the existence of a dynamic sub-cellular population biology. Observations on the proportions of nuclei within mycelia, as well as the allocation of nuclear numbers within conidia and the incidence of post-germination mortality, strongly supported the occurrence of intracellular genomic conflicts. Phenotypic traits normally associated with heterokaryotic mycelia were stably transmitted to homokaryons derived mitotically from heterokaryotic associations, including altered somato-sexual recognition responses. Genetic, epigenetic, and *hyperepigenetic* inheritance systems are proposed to account for these properties.

Conflict was particularly evident in the non-sib-related sympatric and non-sib-related allopatric combinations. Differences in the behaviour of laboratory-synthesized and naturally-occurring heterokaryons suggested that genomic conflict may operate as a key selective force, shaping natural populations. Furthermore, in sympatric populations, co-evolutionary feedback processes may operate to reinforce mechanisms that prevent or limit the take-over of genomic territory by non-self genetic entities. Consideration is given to the options available to fungi for the attenuation of conflict.

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INTRODUCTION

The complex interplay between the key co-ordinates of biological organization, the genotype, phenotype and the environment was once a core concern of population or ecological geneticists. The ideals were embraced within the field of population phenogenetics (Zakharov, 1989) and concepts based upon Waddington's epigenetic landscape. Following the ascendance of "reductionism" over "holism", in one of the great polemical debates of this century, genes have often been considered to be more important than their context in the quest to understand the factors that govern phenotype. Correspondingly, it has become conventional to attribute most phenotypic traits to the presence or action of gene products, with the environment merely providing inputs which set in train a pre-ordained sequence of developmental events. The loss of the wider perspective maybe to our detriment.

The narrower perspective has been reinforced by studies focused on determinate life-forms with defined spatial and temporal boundaries that can be identified as discrete units of selection. However, there are many examples of indeterminate life-forms that possess malleable boundaries which can explore not one, but a host of metastable organizational states (West-Eberhard, 1986).

Some of the organizational states displayed by indeterminate life forms may result from historically optimized or selectively moulded (adaptive) programs which are a reflection of the genetic variation within a population. Other states may be the result of self organizational processes that arise, not because of the properties of the individual components of the system but rather from the integrated boundary / field properties of the system as a whole (Goodwin, 1995; Rayner, 1995; Rayner, in press).

The combination of adaptive strategies and self-organizational processes provides a rationale for the understanding of the dynamic processes underpinning the development of indeterminate systems at a variety of scales (Rayner, 1994a,b,1995). Moreover, the interplay within and between genetic determinacy and epigenetic indeterminacy, engenders conflicts or tension which may lead to evolutionarily creative instabilities or degeneration.

"*Of all the challenges which a fungal mycelium faces during its potentially indefinite life span, the one arguably bring the most powerful combination of risk and promise to the*

selfish genes that it contains is an encounter with another mycelium of the same or different species", Rayner, (1991a).

The following thesis explores this challenge, examining the power of genomic conflict to shape populations and ultimately influence the evolution of genetic exchange systems in organisms with indeterminate life-forms.

Sexual conflicts

Mating necessitates the juxtaposition of two disparate genomes, with differing developmental capacities in the same cell. The genetic information that each encodes provides a framework for the regulation of fundamental cellular events. Any disparities between the genomes with respect to their patterns of transcription, translation and replication may generate untenable conflicts if the complex conditions required to interface their molecular machineries are not resolved.

The act of mating may bring together populations of nuclei and organelles that are capable of forming diverse and potentially unstable relations. Genomic disparity arising within a cell in either a *genetic* sense (as a result of non-self-fusions) or an *epigenetic* sense (as a result of self-fusions between cells in alternative developmental states) engenders the possibility for conflict, coercion, and / or confusion at a cellular level. The incompatibilities that ensue, not only affect the fate of the cells in which they arise, but also the biology of an organism at a variety of hierarchical levels from the subsequent development of its cells, to its population biology and ultimately the delimitation of species.

The association of disparate genetic information within a common cellular domain opens up the possibility for a dynamic social interplay that can perhaps be best understood in terms of the subcellular population biology of the interacting genomes. Such a scenario was envisaged in a mycological context by Pontecorvo in 1946.

"*We may be justified in considering a hypha as a mass of cytoplasm with a population of nuclei. Such a population is subject to: (1) variation in numbers; (2) drift - ie, random variation in the proportion in the different kinds of nucleus; (3) migration - ie, influx and outflow of nuclei following hyphal anastomosis; (4) mutation; (5) selection. There are here*

all of the elements considered by Fisher, Haldane and Sewall-Wright in their works on the genetical theory of populations. No doubt their techniques will be to a large extent adaptable, and extremely useful in, the study of heterokaryotic systems when the time has come for a parallel treatment."

Fungi therefore provide an opportunity, largely unprecedented in eukaryotic organisms, to investigate the consequences of this social interplay. Observed patterns of mating (nuclear and mitochondrial exchange) between fungal individuals within and across the boundaries of interbreeding populations have indicated that invasive genomes may co-exist with, recombine with, replace, or possibly suppress their partners (Coates & Rayner, 1985a; Rayner, 1988; Ainsworth & Rayner, 1989; Ainsworth *et al.*, 1990a).

Genomic conflict: an evolutionary biologist paradigm

A realisation of the importance of disharmony and conflict of the sort displayed by fungi and other eukaryotes following genetic exchange has paved the way for a resurgence in interest amongst evolutionary biologists in the phenomenon of genomic conflict (Hurst, 1992; Hurst, Hurst & Johnstone, 1992). Genomic conflict has been suggested to be the primary driving force behind the evolution of many of the genetic exchange systems that are fundamental to the eukaryotic mode of existence (Hurst, 1992).

Intranuclear conflicts, arising from interactions between selfish genetic elements within a genome that further their own interests at the expense of the genome or cell within which they reside, have been used to account for such diverse phenomena as differential patterns of gene expression (imprinting), the evolution of crossing-over, the production of syncytia during gametogenesis, the presence of multi-copy genes and even the evolution of genome size (Hurst, Hurst & Johnstone, 1992). Moreover, intergenomic conflicts between cytoplasmic genes, and between cytoplasmic genes and nuclear genes, have been proposed to have been important factors in the evolution of sex (Hurst, 1991a); the sexes, (Hoekstra, 1987; Hickey & Rose, 1988; Hurst & Hamilton, 1992), anisogamy (Cosmides & Tooby, 1981; Hurst, 1990; Hastings, 1992; Law and Hutson, 1992), sex ratios (Cosmides & Tooby, 1981; Hurst, 1991b; Hurst & Pomiankowski, 1991; Levy, 1991), and eusociality (Haig, 1992).

The explanatory power of the genomic conflict paradigm is outlined below through a consideration of the evolution of sex, the sexes and anisogamy. Examples are indicated of studies in mycology that may have something new to offer the theories. Other aspects of the theory can be found in the appropriate reviews and will not be discussed further here.

Hickey & Rose (1988) proposed that sex could have arisen from conflicts between ultra-selfish genes and their host genomes, taking the F-plasmid of *Escherichia coli* as a prime example. Any selfish genetic element that 'encourages' fusion between cells, providing the possibility for horizontal transmission, would be more successful than an element that was restricted to a vertical lineage. Hurst (1991a) provided additional support for this hypothesis by highlighting the behaviour of a plasmid residing within the mitochondria of the slime mould *Physarum polycephalum* which promoted the fusion of mitochondria within zygotes, and the formation of recombinant mitochondrial DNA. In the absence of the plasmid no mitochondrial fusion was observed, but when gametes supporting the plasmid were paired with gametes in which it was absent, all mitochondrial progeny from the cross contained the plasmid (Kawano *et al.*, 1991). Mitochondria as 'trapped prokaryotes', may simply represent another F-plasmid scenario, making it unclear whether the phenomenon has any real relevance to the evolution of sex in eukaryotes (Hurst, 1992).

Of considerable importance then, is the discovery by Hänfler *et al* (1992) of a non-mitochondrial plasmid which encodes a protein associated with the hyphal wall of the (+)-mating-type strains of the zygomycete *Absidia glauca*. Artificial heterokaryons or diploids of *Absidia*, constructed by protoplast fusion, produced normal fertile zygospores, but did not produce any detectable quantities of the plasmid protein on their hyphae. The protein cannot therefore be required for sexual differentiation, but may instead be involved with hyphal adhesion or recognition. This plasmid might be the first candidate for a cytoplasmic gene that truly promotes fusion in a eukaryote.

Once gametic fusion had evolved, 'competition' for cellular territory between the different cytoplasmic elements entering a cell generated additional conflicts. Unless resolved these conflicts might lead to extensive cellular degeneration, a situation deleterious to the nuclear genome as well as the interfering cytoplasms.

The avoidance of conflict between cytoplasmic elements was proposed by Hoekstra (1987), and later by Hurst and Hamilton (1992), as the selective impetus for the evolution of sexes, with two as the most stable number. They considered that in a hypothetical sexually out-crossing population that produced isogamous gametes, with no mating types, that any cytoplasmic mutant that could destroy its partners would spread to fixation so long as the costs incurred did not outweigh the potential two-fold transmission advantage. However, some costs would be suffered; most notably, homozygous associations would mutually annihilate each other when the mutant attained high frequencies. Correspondingly, nuclear suppressors would be favoured if they made the organelles with which they were associated incapable of destroying an invader - the benefit to the nucleus would be a reduction in the costs of a cytoplasmic war. Since mutant nuclear suppressor genes would have non-suppressor wild type analogues, heterozygotes would only bear half the cost of suppression and would also avoid the costs inflicted upon a homozygote between two non-suppressors. As a result of this heterozygous fitness advantage a stable heterotic polymorphism would arise. Finally, if an unlinked 'choosy' gene that prevented haploid cells from fusing with cells of the same suppressor type invaded the population, sub-optimal matings would be avoided and a population with binary mating-type genes would arise. This is important, since although exceptions occur in the basidiomycetes, ciliates and some angiosperms, sexes do generally come in twos.

Hurst and Hamilton argued further that systems with multiple mating-types would be intrinsically unstable since they would open the population to invasion by additional selfish elements. Since the slime mould, *Physarum*, possesses at least thirteen different alleles at one of its three mating-type genes (Kawano, Kuroiwa & Anderson, 1987) the mitochondrial fusion plasmid in this organism might be just such an opportunist.

Other examples that depart from the 'normal' patterns of behaviour are also revealing. Two systems of sexual exchange have been shown to operate in a single population of hypotrich ciliates; gametic fusion and conjugation. Sub-populations with multiple mating-types only exhibit conjugation, whilst those with binary mating-types only show gametic fusion (Takahashi, 1977). One inference from such observations could be that multiple mating-types

are maintained in the basidiomycetes (cf ascomycetes with binary mating-types) because sex does not involve fusion (with cytoplasmic mixing) but a form of conjugation (no cytoplasmic mixing).

Law and Hutson (1992) stated that a whole class of nuclear modifier genes could evolve to reduce the transmission efficiency of selfish cytoplasmic elements if they were tightly linked to the mating-type loci. The complex structure of basidiomycete mating-type factors (see Casselton & Kües, 1994, for a review) may be explained in such terms. Furthermore, it is known from studies of illegitimate matings in basidiomycetes that these affect patterns of mitochondrial morphogenesis (Casselton & Economou, 1984) and presumably their replication.

The genomic conflict paradigm also offers an explanation for the evolution of anisogamy (synonymous with the emergence of sexes as opposed to mating-types - Eberhard, 1980; Cosmides & Tooby, 1981; Hurst, 1990; Hastings, 1992; Law & Hutson, 1992). Standard evolutionary theory states that there is no intrinsic cost to sex in an isogamous population, but that a two fold cost is incurred when anisogamy evolves (Maynard-Smith, 1978). Hastings (1992) was able to show that a cytoplasmic mutant could reduce the fitness of a population that it invaded to less that 5% of its original level. Such a large loss of fitness is clearly more deleterious than the maximum two-fold cost of sex imposed by strict uniparental cytoplasmic inheritance and anisogamy. Although the small size of sperm is generally postulated be a panacea for reducing cytoplasmic conflict, however direct evidence for the expected evolutionary linkage between mating-type and gamete size is different to obtain. Two cases have been reported in the mycological literature. Both *Chromocrea spinulosa* and *Sclerotinia trifoliorum* segregate ascospores at meiosis which fall into two distinct size classes, one of each mating-type (Mathieson, 1952; Uhm & Fuji, 1983a,b).

Genomic conflicts are regarded by many as providing a plausible internal driving force behind evolutionary change. Many examples have been sought to support the theories from the literature but no direct tests have been made of its claims. Studies of internuclear conflicts within the domain of a fungal mycelium with multiple mating-type alleles may lend additional credence to its propositions.

Incompatibility systems within fungi

An appreciation of incompatibility systems within fungi has undoubtedly increased the understanding of the potential for generating variation within fungal populations and its impact on their evolution (Esser & Blaich, 1994). Several incompatibility systems that restrict the flow of nuclear genes between individuals have been recognised within the fungi. These may directly preclude the establishment of an sexual phase, or prevent the stable association of nuclei within a common cytoplasm. The two systems of incompatibility that are widely recognised, homogenic and heterogenic, incompatibility are justifiably regarded as key factors affecting the patterns of gene flow within and between populations (Esser & Blaich, 1994) despite misinterpretations resulting from a failure in some cases to clearly differentiate between sex and soma.

Homogenic incompatibility is typically expressed between nuclei that carry identical factors at one (unifactorial-bipolar), two (bifactorial-tetrapolar), or more controversially three (trifactorial-octrapolar) mating-type incompatibility loci (Burnett, 1975). The factors involved vary between the major taxonomic divisions of fungi, but all ensure that the stages required for normal sexual development are not initiated.

Whereas specific mating-typing loci are responsible for homogenic incompatibility, the genetic basis of heterogenic incompatibility is more varied, ranging from interactions between single or multiple nuclear genes to the action of extranuclear genetic elements.

Patterns of genetic exchange in fungi

The filamentous ascomycetes and basidiomycetes exhibit a variety of contrasting life cycles and breeding strategies. Non-outcrossing strategies occur in both, invariably producing clonal offspring, either in the absence in meiosis (amixis/apomixis) or via meiosis between genetically identical nuclei (homomixis/primary homothallism). In the absence of a functional incompatibility system many basidiomycetes can still complete their sexual cycle as homokaryons, producing clonal progeny sets through normal recombinatorial processes.

Evidence for outcrossing and non-outcrossing populations within the same genus, or even within a single species has been described for a number of filamentous ascomycetes. For example, outcrossing populations of *Hypoxylon fuscum*, *H.fragiforme*, *H.mammatum*,

H.rubiginosum, *H.serpens* and *Rosellinia mammiformis* have been found, whereas non-outcrossing was detected in *H.multiforme*, *H.serpens*, *H.purpureum* and *Rosellinia desmazieresii* (Dowson, 1982; Sharland, 1987; Sharland & Rayner, 1986, 1989a,b; Sharland *et al.*, 1988). A similar situation is also found in the basidiomycetes, where some species are entirely outcrossing eg *Stereum gausapatum* and *S.rugosum*; whereas others, eg *Stereum hirsutum*, *S.sanguinolentum*, *S.subtomentosum* and the *S.striatum* complex (including *S.ochraceo-fulvum* and *S.rameale sensu* Jahn, 1971) may contain both outcrossing and non-outcrossing populations that are either partially or wholly isolated (Ainsworth, 1987; Ainsworth *et al.*, 1990a). Other well characterised examples where outcrossing and non-outcrossing populations occur include *Coniophora puteana* (Ainsworth, 1987), *Crinipellis perniciosa* (Griffith & Hedges, 1994), *Heterobasidion annosum* (Korhonen, 1978), *Rhizoctonia solani* (Bolkan & Butler, 1974; Anderson, 1984) and *Sistotrema brinkmannii* (Biggs, 1937; Lemke, 1969; Ullrich, 1973).

Outcrossing strategies require the co-operation of two genetically distinct individuals for completion of events during sexual differentiation (heteromixis or heterothallism). In fully outcrossing ascomycetes, successful mating is based upon complementarity of two alleles at a single mating-type factor (dimixis). In basidiomycetes a number of more complex situations exists. Here each mating-type factor can exist in two or more allelic forms (diaphoromixis). Only the a-mating-type factors of bifactorial (tetrapolar) heterobasidiomycetes such as *Ustilago maydis* and *Tremella mesenterica* possess two alleles (Bandoni, 1963; Puhalla, 1970; Kämper, Bölker & Kahmann, 1994).

Recent additions to the understanding of mating-type locus structure support the view that world-wide more than 12 000 mating-types in *Coprinus cinereus* and 20 000 mating-types in *Schizophyllum commune* might occur (Casselton & Kües, 1994). Diaphoromixis as a breeding strategy can therefore create a colossal bias towards outbreeding at the population level whilst still restricting the incidence of inbreeding between related strains. Detailed studies of mating-type factors have been carried out in a small number of heteromictic mycelial ascomycetes, notably *Neurospora crassa* (*A* and *a* factors), *Podospora anserina* (*mat*$^-$ and *mat*$^+$ factors), and *Cochliobolus heterostrophus* (*MAT-1* and *MAT-2* factors) - see Glass and Nelson (1994)

for a comprehensive review. Molecular genetic studies of homobasidiomycete mating factors have almost entirely concentrated upon two tetrapolar species, *Coprinus cinereus* and *Schizophyllum commune*. The complete sequences and organization of three A-factors isolated from *C.cinereus* have been elucidated (Kües & Casselton, 1992; Tymon *et al.*, 1992; Casselton & Kües, 1994). Studies of the heterobasdiomycete *Ustilago maydis* (Banuett, 1991, 1992; Gillissen *et al.*, 1992; Kamper *et al.*, 1994) have been particularly enlightening since they reveal properties of the homogenic incompatibility loci which are common to both ascomycetes and basidiomycetes.

Molecular analyses indicate that the alleles of the mating-type loci of the ascomycetes studied are genetically dissimilar at the sequence level, and that they are even different sizes. Metzenberg & Glass (1984) therefore refer to the *A* and *a* factors of *Neurospora* as idiomorphs rather than alleles. The factors of all three species examined encode proteins with similarity to the α-box of the yeast transcriptional activator gene MAT-α-1 (Glass, Grotelueschen & Metzenberg, 1990; Debuchy & Coppin, 1992; Arnaise *et al.*, 1993; Turgeon *et al.*, 1993). Other regions have been identified with homology to fungal pheromones. In the a idiomorph only a single regulatory region (*mta-1*) has been identified which has homology to a high mobility group (HMG) DNA-binding domain (Staben & Yanofsky, 1990; Picard *et al.*, 1991; Arnaise *et al.*, 1993; Debuchy, Arnaise & Lecellier, 1993).

Studies of the A-factor in *Coprinus cinereus* have revealed that the A-factors are divided into two complexes corresponding to the α and β subunits already identified by classical recombination studies. Each subunit is composed of three separate genes. The α factor gene encodes a metallo-endopeptidase, and the β factor gene, a small protein of unknown function (Kües & Casselton, 1992; Tymon *et al.*, 1992; Casselton & Kües, 1994). Specificity regions have been identified which encode proteins with motifs characteristic of a group of transcriptional regulators with helix-loop-helix regions (cf Bürglin, 1993). The specificity genes fall into distinct classes, HD-1 and HD-2, each with a separate but conserved homeodomain (Kües & Casselton, 1992). Similar motifs have been found at the Aα locus of *Schizophyllum commune* (Stankis *et al.*, 1992). In addition to the homeodomain, the predicted protein also contains other helices and serine-threonine-proline rich domains. The helices may

be involved with protein-protein dimerization and / or DNA recognition. At their N-termini these helices are reminiscent of the POU domains found on some mammalian transcription factors (Tymon *et al.*, 1992).

Several B-mating-type factors have been cloned from *Schizophyllum commune* (Specht in Casselton & Kües, 1994; Vaillancourt & Raper, 1995). Preliminary sequence data suggested that they contain genes for pheromones and pheromone receptors.

The key events of plasmogamy, karyogamy and meiosis required for successful sexual outcrossing are under complex developmental and genetic controls. Ascomycetes contrast greatly with basidiomycetes in their patterns of genetic exchange, perhaps hinting at underlying differences in their abilities to cope with the conflicts attending the association of disparate genomes in the same cell.

In the majority of well characterised ascomycetes the sexual cycle is initiated by the formation of swollen, often coiled reproductive structures termed ascogonia, which in many species, produce receptive hyphae or trichogynes. Fertilization of the 'female' occurs via one of two routes; gametangial contact or spermatization. In gametangial fertilization 'male' antheridia interact with the trichogynes of the female. Spermatization requires contact of the trichogyne with relatively unspecialized 'male' donor cells such as conidia, microconidia or hyphae. Following contact the acceptor and donor cells fuse and the 'male' nucleus migrates via a fusion pore into the trichogyne.

A range of developmental programs may then be initiated following conjugation which lead to the emergence of fully differentiated structures such as cleistothecia, perithecia, apothecia or loculae. Nuclei divide synchronously to form a small mass of dikaryotic ascogenous hyphae. Karyogamy and meiosis occur within crozier shaped cells that form on the penultimate cell of each ascogenous hypha. Following a variable number of mitotic divisions the recombinant haploid nuclei finally become delimited as spores within an ascus. Mature ascospores are often binucleate as a result of additional mitotic divisions which occur after spore formation.

The life-cycle of heteromictic basidiomycetes is typically partitioned into two distinct phases, the primary or homokaryotic phase, and the secondary or heterokaryotic phase. Primary mycelia typically originate from the germination of genetically pure basidiospores, conidia or

occasionally from the breakdown of a heterokaryon. The mycelium may be coenocytic, with multinucleate hyphal compartments (homokaryons), or septate, with regular cross-walls partitioning the protoplasm into uninucleate compartments (homokaryons / monokaryons).

Secondary mycelia are generated as a result of somatic fusion between two primary mycelia (or possibly a primary and a secondary mycelium) with complementary mating-types. Following fusion of compatible mycelia, nuclei may migrate either unilaterally or bilaterally to establish a more extensive heterokaryotic partnership. In general, mitochondria do not migrate (Casselton & Economou, 1985) so that two distinct heterokaryons are created - though there are exceptions (eg Watrud & Ellingboe, 1973a,b). The nuclei within a heterokaryon may reside within multinucleate hyphal compartments (heterokaryons), or they may be associated as conjugate pairs within single compartments (dikaryons). Some homobasidiomycetes produce diploid secondary mycelia, most notably members of the genus *Armillaria* (Hintikka, 1973; Korhonen & Hintikka, 1974; Ullrich & Anderson, 1978; Anderson, 1982; Franklin, Filion & Anderson, 1983; Peabody & Peabody, 1984 in Korhonen, 1987; Korhonen, 1987;). The progression from diploid to dikaryon to heterokaryon represents a spectrum of creative freedom for the associated partners, each with a different potential for the expression of genetic and developmental variation (Raper & Flexer, 1970).

Specialised cells termed clamp-connections are traditionally associated with the secondary mycelial phase of basidiomycetes (Buller, 1930,1941), but, there are well documented examples where they are entirely absent from the life-cycle, eg some species of *Agaricus* (Elliott, 1978), *Coprinus* (Brunswick, 1924 in Papazian, 1950; Kemp, 1975, 1980a) and *Phellinus torulosus* (Fischer & Bresinsky, 1992). The artificial somatic diploids of *Schizophyllum commune* and the natural diploids of *Armillaria* also do not produce clamps (Koltin & Raper, 1968; Hintikka, 1973; Korhonen & Hintikka, 1974; Korhonen, 1980). Conversely, in some species clamp-connections may be produced on both primary and secondary mycelia, particularly amongst the holocoenocytic basidiomycetes such as *Coniophora*, *Phanerochaete* and *Stereum* (Ainsworth, 1987). It is now apparent that the factors governing their production are complex and that they may be irregular or intermittent.

Consequently their use as diagnostic markers indicating genetic exchange may be unreliable (Stenlid & Rayner, 1989).

Other patterns of nuclear inheritance are also possible in the basidiomycetes including the exchange of genetic material between dikaryons and monokaryons - a process referred to as the Buller phenomenon (Buller, 1930,1931,1941). The Buller phenomenon, has been observed in many fungi eg *Coprinus cinereus* (May, 1988; May & Taylor, 1988), *Coprinus lagopus* (Swiezynski & Day 1960a,b), *Coriolus versicolor* (Aylmore & Todd, 1984), *Echinodontium tinctorum* (Wilson, 1991), *Laccaria bicolor* (Gardes, Wang & Fortin, 1990), *Pleurotus ostreatus* (Vilgalys, Smith & Sun, 1993), *Schizophyllum commune* (Papazian, 1950; Aylmore & Todd 1984), *Stereum hirsutum* (Coates & Rayner, 1985b), *Typhula idahoensis* and *T.ishikariensis* (Bruehl, Jacobs & Machtmes, 1983). Analogous events have also been described between diploid and haploid mycelia of *Armillaria gallica* (Carvalho, Smith & Anderson, 1995) and *A.mellea* (synonym = *A.ostoyae*) (Anderson & Ullrich, 1982; Guillaumin, Anderson & Korhonen, 1991; Rizzo & Harrington, 1992; Rizzo & May, 1994).

When both nuclei of a heterokaryon are compatible with a homokaryon, one of the component genotypes may be preferentially selected. This has been described in *Laccaria bicolor* (Gardes, Wang & Fortin, 1990), *Pleurotus ostreatus* (Nguyen & Niederpruem, 1984), *Schizophyllum commune* (Crowe, 1960; Ellingboe & Raper, 1962) and *Stereum hirsutum* (Coates & Rayner, 1985b). Occasionally nuclear replacement is observed where both nuclei of a heterokaryon invade and take over domain originally occupied by a homokaryon (Crowe, 1960; Ellingboe, 1963; Coates & Rayner, 1985b; May, 1988). Similar replacement reactions, with complex outcomes, have also been described in diploid - haploid interactions amongst various species of *Armillaria* (Carvalho *et al.*, 1995; Rizzo & Harrington, 1992; Rizzo & May, 1994).

Nuclear replacement reactions have also been detected in self-paired homokaryons and dikaryons of *Coriolus versicolor* and *Schizophyllum commune* (Aylmore & Todd, 1984) and in *Chondrostereum purpureum* (Ainsworth, unpublished). They have not been seen in fungi with multinucleate hyphal compartments eg *Phanerochaete velutina* or *Stereum hirsutum*

where the lytic reactions that follow non-self-fusions are more rapid than those observed in the dikaryons (Ainsworth & Rayner, 1986).

Taken together, observations of this kind led Rayner *et al* (1984) to propose that heterokaryosis depended upon an interplay between three basic processes; access migration, acceptor migration and stabilization. Access migration allows the ingress of donor nuclei into an acceptor mycelium at a rate determined by the level of genetic similarity between the donor and the acceptor. Acceptor migration proceeds at a rate determined entirely by the pre-existing architecture of the recipient mycelium; consequently different invasive nuclei can migrate at the same rate. Heterokaryotic mycelia frequently degenerate or breakdown into one, or both, component genotypes if mechanisms that promote stability are not in operation. Stabilisation requires the override of developmental incompatibilities that are potentially a feature of any associations between nuclei of disparate origin.

Operating alone, access migration produces appressed mycelia with reduced marginal extension. Since inward radial migration is impeded relative to outward lateral migration, heterokaryotic zones with a distinctive "bow-tie" appearance may be formed (Coates, Rayner & Todd, 1981; Coates & Rayner, 1985a).

A failure to stabilize can lead to the replacement of the acceptor nuclei, an outcome that has been observed in the 'bow tie' reactions of both *Stereum hirsutum* (Coates, 1984) and *Stereum gausapatum* (Boddy & Rayner, 1982). 'Bow-ties' may be halted by the expression of somatic incompatibility responses. The bow-tie region might therefore result from conflict between acceptor / stabilisation functions, implying that override is largely a function of resident, not invasive genomes.

Heterogenic incompatibilities

Repulsion between interacting mycelia was first described by Reinhardt in 1892 (Esser & Blaich, 1994). Later, Vandendries applied the term "barrage" to describe this phenomenon, which many consider to be synonymous with the expression of heterogenic incompatibility (Esser & Blaich, 1973, 1994; Esser & Meinhardt, 1984). The recent classification of heterogenic incompatibility responses by Esser & Blaich (1994) into four categories: mutual

intermingling, inhibition, mutual intermingling followed by inhibition or mutual repulsion is somewhat superficial. A more systematic understanding may arise if a distinction is made between incompatibility responses that are expressed pre- or post-fusion, and between those that operate as a result of genetic or physiological disparity. In particular, clear distinctions need to be made between events that restrict hyphal fusion *per se* (cf self incompatibility in *Fusarium* - Correl, Klittitch & Leslie, 1989; Leslie, 1983) and those that operate post-fusion, since the former may be best termed heterokaryon incompatibility, and the latter somatic incompatibility. Considerations of heterogenic incompatibility responses elicited between homokaryons and those expressed between heterokaryons also need to be distinguished - Esser & Blaich, 1994, often interpreted the two in the same light.

Heterogenic incompatibility in Ascomycetes

Heterogenic incompatibility was first explicitly documented between strains of *Podospora anserina* derived from different geographical locations (Rizet, 1952, 1953; Rizet & Esser, 1953; Esser, 1954). Since then heterogenic incompatibility has been described in all of the fungal groups and also amongst other eukaryotes (Esser & Blaich, 1973). A genetic basis for heterogenic incompatibility was revealed in a study of two races of *Podospora anserina*, *s* and *M* (Rizet & Esser, 1953; Esser 1954, 1956). Two mechanisms were identified. The first an allelic mechanism with two loci, *t* and *u*, did not interfere with sexual compatibility *per se* - rather allelic differences at one or both loci led to the expression of a somatic rejection response when strains were paired together. The second mechanism was non-allelic and depended upon the interaction of two specific alleles at two different loci. For example, in an interracial cross between *s* and *M*, four loci *a, b, c* and *v* exhibited incompatibility in the combinations a_1 / *b* and c_1 / *v*. By examining additional interracial crosses and by following the behaviour of strains altered by mutagenesis, a further six heterogenic incompatibility loci, one allelic and five non allelic were identified (Bernet, 1967; Delettre & Bernet, 1976). Belcour (1971), identified a mutant *minor (mi)*, which mapped very closely to the *e* heterogenic incompatibility locus and which made a product incompatible with the product of the *c* locus. A suppressor of the *mi*

mutant was also described which mapped to the *c* locus, perhaps indicating that the 'fit' of the two gene products could be restored by a mutational adjustment at the second locus. Suppression of incompatibility by dihydrostreptomycin later indicated that the protein complexes involved might be ribosomes (Bernet, Bégueret & Labarère, 1973).

Heterokaryons isolated from single ascospores were unstable when the allelic mechanism of incompatibility operated, sectoring into homokaryons of both nuclear types. In heterokaryons where the non allelic mechanism operated, no sectoring was observed because one genotype was eliminated (Esser, 1956, 1959). Furthermore, it was elucidated that the *b* allele was aggressive towards the a_1 allele.

Glass and Kuldau (1992), sequenced two clones of the *b* locus which were originally isolated by Turcq (1989). Both contained open reading frames which only differed at a few amino acids. Similarly, detailed studies of the *het s* and *het S* alleles revealed open reading frames encoding proteins of 289 amino acids which only differed at fourteen positions (Deleu, Clavé & Bégueret, 1993). Moreover, in a study of thirteen wild strains it was found that only a single amino acid difference was required for the expression of an incompatibility response.

Heterogenic incompatibility has also been investigated in other ascomycetes. Recent estimates suggest that at least eleven *het* loci are found in *Neurospora c*rassa (Kuldau & Glass, 1994) of which at least one, het *c* supports multiple alleles (Perkins, 1968). In contrast to *Podospora* and *Neurospora*, heterogenic incompatibility within *Aspergillus* does not prevent heterokaryon formation or any subsequent sexual compatibility (Butcher, 1968), rather the heterokaryons show weak growth, and might be at a selective disadvantage. Such incompatibility was first described in *Aspergillus niger* (Gossop, Yuill & Yuill, 1940) and in *A.fonsecaeus* (Raper & Fennell, 1953). Later investigations confirmed its occurrence in a range of other anamorphic species (Caten, Butcher & Croft, 1971). Studies on teleomorphic species such as *A.glaucus* (Jones, 1965), *A.heterothallicus* (Kwon & Raper, 1967) and *A.nidulans* (Grindle, 1963a, 1963b; Jinks *et al.*, 1966; Butcher, 1968; Croft & Jinks, 1977; Dales & Croft, 1977, 1980) finally provided the opportunity to establish a genetic component for the incompatibility.

Eight *het* loci were identified in *A.nidulans* with an allelic mechanism of incompatibility. These were distributed between five of the eight chromosomal linkage groups. Two of the loci, *het B* and *het C,* were multi- allelic (Dales & Croft, 1980). It is not known if the *het* genes identified on specific chromosomes corresponded for different strains, or whether they differed (Dales, Moorhouse & Croft, 1983; Dales & Croft, 1990).

The *het* genes were grouped into two broad classes by Croft *et al* (1980) according to their effects in protoplast fusion experiments. In the first class, *het A, C, D* loci, heterokaryotic fusion products containing different alleles were indistinguishable from those which were homo-allelic. The second class, *het B, F* loci, produced heterokaryons which were morphologically abnormal. In all cases fusions between hyphae led to the expression of incompatibility. Heterogenic incompatibility therefore arises from a number of mechanisms which may directly prevent successful fusion (*het A, C, D*) or by affecting post-fusion development (*het B, F*) (Croft, 1985).

Heterogenic Incompatibility in Basidiomycetes

Homokaryons of the basidiomycete *Hirschioporus abietinus* collected from North America could be divided into two incompatibility groups (Macrae, 1967). Paradoxically, both groups were compatible with homokaryons obtained from Europe. Fischer & Bresinsky (1992) discussed similar intersterility phenomena in *Phellinus torulosus* which they linked to geographical separation, and more tentatively to host specialization. Homothallic, bipolar and tetrapolar forms of *Sistotrema brinkmannii* have been identified (Biggs, 1937; Lemke, 1969; Ullrich, 1973) which show complete sterility between the bipolar and the tetrapolar forms. Both, however, contain intersterile subgroups. Neither geographical separation, nor substratal preference, could account for the intersterility groups in the tetrapolar forms, but substrate preference was apparent amongst the different bipolar intersterility groups (Ullrich, 1973).

Cryptic speciation, potentially attributable to heterogenic incompatibility has been reported in a number of other basidiomycetes including *Armillaria* (Anderson, Korhonen & Ullrich, 1980), various members of the *Corticiaceae* (Hallenberg, 1984, 1988), *Exidiopsis plumbescens*

(Wells & Wong, 1989), *Hirneola auricula-judae* (Barnett, 1937), *Mycocalia denudata* (Burnett & Boulter, 1963), *Pleurotus* (Hilber, 1978), *Rhizoctonia solani* (Anderson, 1984) and *Serpula himantioides* (Harmsen, 1960). Interracial incompatibilities have also been observed within a number of heterobasidiomycetes including *Ustilago violacea* (Bauch, 1927; Vandendries, 1927, 1929), *Ustilago avenae* and *U.laevis* (Grasso, 1955). In many cases it is not known whether the origins of the incompatibilities are homogenic or heterogenic (Esser & Blaich, 1973).

A genetic basis for heterogenic incompatibility between homokaryons of a number of basidiomycetes has been revealed. In *Polyporus ciliatus* the formation of a barrage was directly attributed to the interaction of three genes. Barrage formation required the presence of the bi^+ allele in at least one mating partner, and allelic heterogeneity at two other loci, *bfI* and *BfII*. Heterozygosity at a single locus (B-factor) has been implicated in the formation of a wide band of stationary or migrating appressed mycelium in interactions between homokaryons of *Stereum hirsutum* (Coates & Rayner, 1985a). This may be analogous to the homogenic incompatibility attributed to B-factor mating-types, indicating a dual role for this locus (cf A-mating-type of *Neurospora*). Heterogenic incompatibility is also responsible for a complete or partial isolation of at least two of the three host specialised (S,P,F) populations of *Heterobasidion annosum* (Korhonen, 1978; Korhonen *et al.*, 1989; Capretti *et al.*, 1990; Stenlid & Karlsson, 1991). Five loci (S,P,V_1,V_2,V_3) have been described in North American populations (Chase & Ullrich, 1990a, 1990b). Two strains are only compatible if both possess the (+) allele in at least one of the loci. A census revealed that North American S populations were S^+,P^-,V_2^- and +/- at the remaining loci, whereas P populations were S^-,V_2^+ and contained +/- alleles at the P,V_1,V_3 loci.

Genetic dissimilarity influences both the intensity and frequency with which somatic incompatibility responses are elicited between many, but not all basidiomycete homokaryons. A direct relationship, with more distantly related strains rejecting the most strongly was found in *Rhizoctonia solani* (Anderson, 1984) and *Stereum hirsutum* (Coates, 1984). Conversely, Punja & Grogan (1983) found no correlation between relatedness and rejection frequency in *Sclerotium rolfsii*.

The operation of two systems of incompatibility between homokaryons of mycelial fungi results in a delicate balance between the evolutionarily creative tendencies for acceptance (collectivism) and rejection (individualism) (Rayner, 1991a). The success or failure of a mating can be regarded as the outcome of the interplay between these opposing forces, one system overriding (Rayner & Todd, 1979; Rayner *et al.*, 1984) or bypassing (Brasier, 1984) the other.

Rayner *et al.*(1984) proposed that outcrossing in basidiomycetes normally requires mechanisms that override heterogenic incompatibility until such a time as a stable secondary mycelium emerges. Heterogenic incompatibility systems operating between heterokaryons may then prevent further nuclear entry and migration (Rayner & Todd, 1979).

Incompatibility responses expressed between secondary mycelia largely, though not entirely, preclude the exchange of genetic information. Early reports of somatic incompatibility between heterokaryons include the formation of barrages between strains of *Fomes cajanderi* (Adams & Roth, 1967), *Fomes ignarius* (Verrall, 1937), *Fomes pinicola* (Schmitz, 1925; Mounce, 1929), *Oudemansiella radicata* (Campbell, 1938), *Phaeolus schweinitzii* (Childs, 1937) and *Poria weirii* (Childs, 1963).

Little is known of the genetics of somatic incompatibility, however multi-allelic major genes are thought to be involved (Rayner, 1986; Kay & Vilgalys, 1992; Hansen, Stenlid & Johansson, 1993). The intensity of rejection responses between secondary mycelia varies according to their overall level of genetic similarity and according to the particular species under consideration (Adams & Roth, 1967; Barrett & Uscuplic, 1971; Todd & Rayner, 1978; Adams *et al.*, 1981; Boddy & Rayner, 1982; Rayner & Turton, 1982; Coates, 1984). Backcrossing experiments in a number of species have revealed that in near isogenic lines the number of loci responsible can be reduced to a handful, however the relevance of such studies to interactions between naturally-occurring heterokaryons is not clear.

Genetic factors *per se*, may not be entirely responsible for many examples of incompatibility. Environmental conditions and the organizational status of the mycelium are also factors known to influence both the rapidity and intensity with which rejection responses are elicited (Stenlid & Rayner, 1989). Barrett & Uscuplic (1971) for example observed both quantitative and

qualitative changes in the expression of incompatibility responses between heterokaryons. Repeated subculturing of *Phaeolus schweinitzii* gradually led to a reduction in the frequency with which rejection occurred. Moreover, subcultures taken from either side of interaction zones, when repaired produced less obvious barrage zones than in the original pairing. Similar observations have been made for interactions between strains of *Stereum* (Coates & Rayner, 1985a; Rayner, 1986) and *Lentinus edodes* (Kinugawa & Inoue, 1977), where an increased tendency to reject was noted in pairings between 'aged' strains.

Rayner (1991b) hypothesized that the formation of degenerate zones may be a consequence of interactions between any non-linear systems with multiple attractors - essentially "two's company, three's a crowd". Associations between three or more entities are universally unstable (Kauffmann, 1993) consequently genomic symbioses should perhaps be regarded as dynamical systems balanced at the edge of chaos.

Alternative incompatibility systems

Incompatibility in the sense of Esser & Blaich (1994) is largely concerned with all or nothing events. Either two strains can mate, or they cannot. Either they can form heterokaryons, or they cannot. However, fungal mycelia are capable of exhibiting a wider spectrum of compatibility / incompatibility responses than can be simply encompassed by the terms, plus and minus. Incompatibility *per se*, is indeed an all or nothing event, but there is a strong need to recognise the fine balance that exists between the opposing tendencies of acceptance and rejection, association and dissociation, communication and suppression, competition and co-operation. The dilemmas that such choices engender are better described by the term conflict than incompatibility, and this is the sense in which this term is used in the remainder of the thesis.

Numerous less clear cut interactions have been described in the fungi which have not been explicitly outlined as incompatibilities, but which may implicate genomic conflicts. Conflict might arise through allelic disparities or heterozygosity in almost any gene give the appropriate genetic environment or context.

Ryan and Lederberg (1946) described a *leucine* - requiring mutant of *Neurospora crassa*, that in association with a near isogenic revertant, produced heterokaryons composed almost entirely of mutant nuclei. When in combination with wild type strains such the non-adaptive changes in ratio were not seen (Ryan, 1946). Similar findings have been made for heterokaryons of other species (Tuveson & Garber, 1961; Puhalla & Mayfield, 1974; Typas & Heale, 1976, Wiebe *et al.*, 1992). Davis (1966) speculated that all heterogenic conflict is a pleiotropic effect of genes with other functions, and that evolutionary diversification would in itself lead to heterokaryon incompatibilities whether heterogenic incompatibility is the cause, or the consequence, of such diversification is not really clear.

The notion that conflict can be a strain specific phenomenon is emphasized by work on the interactions between *m* and *pan-1* mutants (Davis, 1960a,b) and auxotrophic strains of *Neurospora crassa* (Holloway, 1953). In the latter example when three auxotrophic strains with the same mating-type (A) were paired in all possible combinations, all produced stable heterokaryons. However, only 54 out of 264 crosses resulted in stable heterokaryons when ascospores generated from the three auxotrophs and a single wild type strain were back-crossed with the original parental auxotrophs.

The fact that disparity at almost any locus can give rise to conflict is emphasized by studies on UV-light, X-ray, γ-ray and N-methyl-N'-nitro-N-nitrosoguanidine induced mutants of *Aspergillus nidulans* (Dales & Croft, 1980). Mutants with altered incompatibility responses were found to map to various positions throughout the genome, but none of them were located at any of the known *het* loci described in this fungus.

Genomic conflicts are also likely to arise from a host of other sources. Conditional recessive lethals for example, which are only activated during specific developmentally regulated transitions, could be easily maintained within a population, generating conflict (cf better studied unconditional recessive lethals). Leslie & Raju (1985) found that most wild type strains of *Neurospora crassa* carried one, or more, recessive genes that when homozygous in a heterokaryon were detrimental to fertility. Overall, in a sample of 80 natural strains, 106 recessive genes were identified that impaired sexual development (Leslie, 1985).

Chromosomal instabilities are also common in fungi (cf Fincham, Day & Radford, 1979). Extensive studies of *Neurospora* have failed to detect any that directly influence fertility or patterns of nuclear exchange (Perkins & Turner, 1988). However, nuclei that fuse in somatic tissues to produce transient diploids may progressively dissociate to produce genetically variable aneuploids. Such events could be considered as a means to avoid conflict (cf inheritance of chromosomes and mitochondria in mouse / human hybridoma line - Ziegler & Davidson, 1981).

Meiotic drive genes have featured strongly in discussions of the role of genomic conflict in evolutionary biology (Hurst, Hurst & Johnstone, 1992). The conflicts that they engender may be expected to lead to an arms race between driving genes and modifiers in sensitive genes. Spore killer genes are relatively well documented in fungi and have been described in *Podospora anserina* (Padieu & Bernet, 1967), *Fusarium moniliforme* (Kathariou & Spieth, 1982) and *Neurospora* (Perkins & Turner, 1988). Following world-wide studies of the *Sk-2*^{k} and *Sk-3*^{k} alleles of *Neurospora* their distribution was found to be highly restricted (Perkins & Turner, 1988). Most wild type strains tested were sensitive to the killer alleles and only a limited number of resistant strains were detected (Turner, 1977). Surprisingly the killer alleles were not replacing their sensitive counterparts, perhaps reflecting an additional cost to their activity, or to hidden levels of resistance in the wild.

Homokaryotic genotypes recoverable form heterokaryotic mycelia commonly deviate from 1:1. Asymmetries of this nature, either in the underlying mycelium, or arising from differential post-association viability, will have significant consequences for interactants. Contributions to both the expression of phenotype and to the future gene pool will be affected by such inequalities and are consequently a major source of genomic conflict.

Nuclear ratios within mycelia

A large number of ascomycetes harbouring asymmetric nuclear ratios following de-heterokaryotization have been described including *Ascochyta imperfecta* (Sanderson & Srb, 1965), *Aspergillus nidulans* (Warr & Roper, 1965; Clutterbuck & Roper, 1966), *Cephalosporium mycophilum* (Tuveson & Coy, 1961), *Fusarium graminearum* (Wiebe *et al.*,

1992) and *Fusarium oxysporum* (Buxton, 1954), *Gibberella fujikuroi* (Sidhu, 1983a,b), *Neurospora crassa* (Beadle & Coonradt, 1944; Atwood & Mukai, 1953; Pittenger, Kimble & Atwood, 1953; Prout *et al.*, 1953; Pittenger & Atwood, 1954,1956; Davis, 1959,1960a,b; Pittenger & Brawner, 1961; Mullaney & Papa, 1982), *Pencillium cyclopium* (Jinks, 1952; Rees & Jinks, 1952) and various *Verticillium* species (Tuveson & Garber, 1959,1961; Coy & Tuveson, 1961; Puhalla & Mayfield, 1974; Typas & Heale, 1976). In most cases, ascomycetes do not tolerate significant genetic disparities within their heterokaryons, consequently by far the greatest levels of conflict should be apparent when the nuclear ratios within basidiomycete heterokaryons are examined. Unfortunately, in comparison to the ascomycetes, relatively few studies have examined nuclear ratios within basidiomycetes.

Strict dikaryons should not be expected to exhibit any significant departure from a 1:1 nuclear ratio; but this has been found to be the case in a number of studies. Asymmetries as high as 4000:1 in favour of one component of a heterokaryon have been detected in *Schizophyllum commune* (Snider, 1963; Snider & Raper, 1965). Similar results in this 'model' basidiomycete, using completely different methods to recover the 'neohaplonts', were later reported by Wessels *et al* (1976) and Raper (1985). However, relatively unbiased genotype recovery rates have also been reported in *Schizophyllum commune* (Papazian, 1950; Miles & Raper, 1956; Snider & Raper, 1965), as well as in other dikaryotic basidiomycetes such as *Coprinus cinereus* (Raper & Raper, 1966), *Coriolus versicolor* (Todd & Rayner, 1978), *Flammulina velutipes* (Brodie, 1936) and *Pleurotus ostreatus* (Leal-Lara & Hummel, 1982; Kay & Vilgalys, 1992). Several other studies on dikaryons have indicated significant departures from 1:1, including *Coprinus macrorhizus f. microsporus* (Takemaru, 1964), *Flammulina velutipes* (Aschan, 1952; Fries & Aschan, 1952; Aschan-Åberg, 1960; Kemp, 1980b), *Lentinus edodes* (Nishibori & Kinugawa, 1978; Kinugawa, 1977) as well as *Pholiota nameko* (Arita, 1979), making it difficult to generalize about the situation in dikaryons.

Great care therefore needs to be taken when interpreting such conflicting reports, since the asymmetries may be generated in a number of ways. In particular, the mode of de-dikaryotization may strongly influence the results obtained. Some approaches, especially those using metabolic poisons, are very harsh and may significantly influence the survivorship of

susceptible genotypes (Kerruish & DaCosta, 1963; Leal-Lara & Hummel, 1982). A clear example of biased recovery that may not indicate underlying inequalities is provided by the conflicting reports from studies of *Flammulina*. Aschan (1952), Åschen-Aberg (1960) and Kemp (1980b) all found that only a single genotype could be recovered from dikaryons of *Flammulina velutipes*. Kemp (1980b) provided an explanation for this when he noticed that the majority of oidia in *Flammulina* are formed from the nucleus that divides in the main hyphal axis, and not from nuclei dividing in the clamp-cell itself. The asymmetric recovery of one genotype cannot therefore be linked with certainty to an underlying ratio disparity as it has been in many other studies. On the other hand, whatever the cause, the population consequences of such apparent ratio asymmetries remain the same.

An alternative mechanism, leading to asymmetry is that one component of a dikaryon may become inviable as a result of the past interactions with its partner or cytoplasm. Microsurgical studies on *Galerina* and *Hirschioporus* (Harder, 1927a, Fries & Aschan, 1952) revealed that half of the strains recovered were of one mating-type and the remainder produced mycelia which ceased growth or died. Fries & Aschan (1952) suggested that the interaction between the nuclei within the dikaryon generated disharmony between the cytoplasm and the invasive nucleus during dikaryon formation. Sadly this was not followed up.

Studies on multinucleate heterokaryons have also revealed asymmetries. Established heterokaryons of *Rhizoctonia solani* (Anderson, 1982) contained unbiased nuclear populations, but prior to their establishment and stabilization, the ratios were often skewed in favour of the 'maternal' nuclei. Furthermore, the heterokaryons generated appeared more like the 'maternal' parent - a similar claim has also been made for common-A heterokaryons *Schizophyllum commune* (Raper, 1985). The frequency of recovery of the component genotypes from common-A heterokaryons of *S.commune* fluctuated (Snider & Raper, 1958) and nuclear ratios within heterokaryons of *Agaricus bisporus* were subject to directional changes over time (Wang, 1980). Dickhardt (1985) later found that the nuclear ratios within mycelia of *Agaricus bisporus* and *A.bitorquis* varied both with the age of the mycelium and the method of 'neohaplont' generation. These observations raise important questions about the stability of nuclear ratios within mycelia.

Pontecorvo (1946), suggested that selection could be responsible for adaptive responses shown by heterokaryons in response to environmental uncertainties. Rees & Jinks (1952) also concluded that heterokaryons of *Penicillium* could respond adaptively as a result of the selection of the most fit nuclear ratios. Similar claims have also been made for *Agaricus bisporus* (Wang, 1980), *Aspergillus nidulans* (Warr & Roper, 1965), *Gibberella fujikuroi* (Sidhu, 1983a, 1983b), *Verticillium albo-atrum* and *V.dahliae* (Typas & Heale, 1976).

Clutterbuck & Roper (1966) reported that interhyphal variations in nuclear ratios in *Aspergillus* were correlated with extension rate. They inferred that hyphae with optimal ratios would grow faster than their neighbours and would be favourably maintained at the margin. On the other hand, *Neurospora* heterokaryons retained stable nuclear ratios under a wide range of conditions and extension rates (Pittenger & Atwood, 1956), which Clutterbuck & Roper (1966) ascribed to the extensive nuclear mixing that occurs for at least 1cm behind the growing margin (Ryan, Beadle & Tatum, 1943). Local adaptations that did arise through selection or drift, were thought to have been counteracted by such mixing. On the other hand, Buxton (1954), took a contrasting view, arguing that the limited cytoplasmic mixing he observed in *Fusarium*, would lead to the drift of a nuclear population towards a homokaryotic state, thereby preventing adaptations from occurring.

Apparently non-adaptive changes in nuclear ratio have also been observed. For example, balanced heterokaryons of *Verticillium* supporting a nuclear genotype with a nutritional deficiency breakdown upon sub-culturing to produce heterokaryons with highly disparate ratios in which the mutants predominate (Puhalla & Mayfield, 1974). Such a change is difficult to explain in terms of the adaptive selection hypothesis.

Systematic changes in nuclear ratios have been described in some strains of *Neurospora* (Ryan & Lederberg, 1946; Holloway, 1955; Davis, 1960b). A satisfactory explanation, hinting at a simple genetic basis, awaited the work of Pittenger & Brawner (1961). The majority of single conidium derived mycelia that they examined produced heterokaryons with nuclear ratios corresponding to those expected from a consideration of the allocation of nuclear numbers to conidia ie 1:1, 1:2, 2:1, and less often 1:3, 3:1, 2:3, 3:2, 1:4 and 4:1. However, some of the heterokaryons they examined produced conidium-derived mycelia with the more

disparate nuclear ratios at a frequency greater than expected. In these strains, nuclei carrying a weakly dominant *I*-allele inhibited the multiplication of nuclei with an *i*-allele. When the proportion of *i* nuclei exceeded 70%, the *I* nuclei were unable to exert their dominance, producing heterokaryons with normal nuclear ratios.

The genetical basis for nuclear ratio asymmetries has also been described in at least one basidiomycete (Raper, 1985). In fully compatible dikaryons of *Schizophyllum commune* the recovery of genotypes could be linked directly to the alleles possessed by the associated nuclei at their B-factor mating-types loci. The alleles were ranked in a dominance hierarchy (*B14* > *B51* > *B35* > *B41* > *B42* > *B4*). The identity of the A factor had little effect. Further work indicted that the hierarchy was in fact controlled by the Bβ allele and not the Bα allele. Mutants with constitutive B-factor expression had lower competitive abilities than normal, whereas B-factor deletion mutants had higher competitive abilities.

Direct evidence for genomic conflict has been provided in a study of the rates of recovery of homokaryotic and heterokaryotic colonies from heterokaryons of the psuedohomothallic gasteromycete *Mycocalia denudata* (Burnett & Boulter, 1963). When media supporting maximal spore germination were used, virtually all of the colonies obtained were homokaryotic (76 - 80%). This finding has one of two explanations.

Firstly the allocation of nuclei to spores could have deviated from that expected by chance, perhaps as a result of a mechanism that favoured the co-segregation of nuclei with the same mating-type. However, this is unlikely since it would require a reversal of the trend found in most pseudohomothallic fungi, which either show a random allocation of nuclei to spores (Elliott & Challen, 1983) or a bias in favour of the co-segregation of nuclei with different mating-types (Sass, 1929; Burnett, 1956; Kennedy & Burnett, 1956; Beckett & Wilson, 1968). The alternative explanation is based upon internuclear conflict within heterokaryotic spores.

Homokaryotic spores only contain one type of nucleus and should not therefore be subject to conflict. In contrast, nuclei within heterokaryotic spores are genetically distinct and so may be prone to incompatibilities of a nature described in preceding sections. Such incompatibilities could lead to a variety of developmental deficiencies, including a failure to germinate. Both the

overall levels of germination (approximately 40%) and the predominant recovery of homokaryons of both types in equal proportions strongly supports this suggestion.

The Present Investigation

The following thesis adapts theories arising from the genetical theory of populations to examine the conjecture that genomic conflicts occur within fungal mycelia and that these may be important determinants of population structure.

The issues raised in this introduction are addressed in the context of naturally-occurring and laboratory-synthesized heterokaryons and homokaryons of two holocoenocytic basidiomycetes, *Heterobasidion annosum* and *Stereum hirsutum*. Using a combination of fluorescence microscopy, molecular DNA fingerprinting and laboratory-based pairing experiments, nuclei and mitochondrial genomes are followed through a variety of sib-related, non-sib-related sympatric and non-sib-related allopatric associations. Patterns of genetic exchange are examined along with investigations into the allocation of nuclei to hyphae and conidia. The somato-sexual responses of homokaryons recovered from heterokaryons via conidia, protoplasts and basidiospores, and the influence of different combinations of genomes upon spore germination and post germination mortality, are all examined. The importance of nucleo-cytoplasmic interactions affecting self-non-self recognition responses are also considered.

The thesis itself is organized in a fashion reflecting a personal journey, which was ideological rather than spatial or temporal. It begins by outlining studies on the population dynamics of nuclei within heterokaryotic mycelia. Much of the information provided was originally collected as the 'bread and butter' data for the enumeration of nuclear numbers within laboratory-synthesized heterokaryons. However, it became clear that much of this information was of value in its own right. The detection of asymmetric nuclear ratios was not a great surprise, but variations in the allocation of nuclear numbers to conidia, the occurrence of post-germination mortality, and differences in the rates of recovery of heterokaryotic conidia compared to the predictions were not expected - section I. The question of how much of this variation was attributable to the genomic interactions *per se*, and how much was environmentally regulated led to the investigation of nuclear ratios under different conditions -

section II. The wider significance of genomic conflict was emphasized when the behaviour of allopatric heterokaryons of *Heterobasidion annosum* were compared to the data obtained in sympatric and naturally-occurring heterokaryons- section III. The notion of conflict (uneasy co-existence), as a phenomenon distinct from incompatibility (absolute non-integration), arose from the finding that the incidence of rejection, and its degree were dependant upon factors other than those located at the mating-type loci, and that epigenetic inheritance systems could modify (moderate) the behaviour of genomes in an association - section IV. The switch to studies on *Stereum* - sections V and VI was made in order to examine the validity of the findings in *Heterobasidion* and to examine the basic phenomenon of self-non-self recognition in a system with more readily assessed compatibility - incompatibility responses.

The thesis ends with a consideration of where other examples of conflict might be found within the fungi, and expands upon the systems of attenuation that are available to fungi to avoid conflict - section VII.

SECTION I

DISTRIBUTION PATTERNS OF NUMBER OF NUCLEI IN CONIDIA FROM HETEROKARYONS OF *HETEROBASIDION ANNOSUM* (FR.) BREF. AND THEIR INTERPRETATION IN TERMS OF GENOMIC CONFLICT

INTRODUCTION

Heterobasidion annosum is a polyporaceous wood decay basidiomycete which causes serious root and butt-rot in a variety of coniferous trees in northern temperate forests (Hodges, 1969). Mating compatibility in *H.annosum* is determined by the possession of different alleles at a single genetic factor, indicative of a unifactorial or bipolar mating-type system (Korhonen, 1978; Holt, Gockel & Hüttermann, 1983). This system ensures that outcrossing occurs in 50% of sib-related pairings and virtually 100% of non-sib related pairings due to the multi-allelic nature of the factor within a population (Raper, 1966). Recently gene flow within, and between three 'host-specialized' intersterile races, ie Spruce (S), Pine (P) and Fir (F), has been examined (Korhonen, 1978; Capretti *et al*, 1990) A fourth, putatively non-outcrossing, non-pathogenic population, assigned to a new species, *Heterobasidion araucariae*, occurs in the southern hemisphere (Buchanan, 1988).

H.annosum exhibits holocoenocytic nuclear behaviour (Boidin, 1971), characterized by the presence of multinucleate hyphal compartments in both homokaryotic and heterokaryotic mycelia. Homokaryotic and heterokaryotic cultures of *H.annosum* produce abundant mitotically derived conidia, which may be either homokaryotic or heterokaryotic when originating from a heterokaryotic mycelium (Korhonen, 1978; Hsiang, Edmunds & Driver, 1989). It may be postulated that the recovery rates of different homokaryotic genotypes from conidia of a heterokaryon will directly reflect the underlying nuclear ratios in the mycelium, once the effects of conidial nuclear number distribution and differential spore viability have been taken into account. Alternatively, if nuclei are not distributed randomly into conidia this may provide evidence for the preferential incorporation of one genotype at the expense of the other. Conidiation in *H.annosum* therefore allows a direct assessment of the influence of genomic conflict on an individual nuclear genotype in a variety of heterokaryotic associations.

Associations between distantly related genomes (occurring for example when allopatrically derived organisms are mated in the laboratory) may be expected to exhibit maximal disharmony, leading to various kinds of developmental conflict, emergent incompatibility, instability and ultimately, protoplasmic degeneration. Indeed this has been demonstrated not only in the fungi (Ainsworth & Rayner, 1989; Ainsworth *et al*, 1992), but also for a variety of

other taxonomically diverse organisms (Zakharov, 1989). However, the possibility that genomic disparity may also have important effects on individual fitness within sympatric populations has received relatively little attention.

The study described here therefore examines the effects of genomic disparity on certain aspects of mitotic spore production and germination in sympatically derived heterokaryons of an economically important basidiomycete, *Heterobasidion annosum* (Fr.) Bref. (synonym, *Fomes annosus* (Fr.) Karst.)

MATERIALS AND METHODS

Strains and culturing

Unless otherwise stated isolates were grown at 20°C in 90mm diameter unvented Petri dishes containing approximately 15 ml malt extract agar - (MEA: 20 g l^{-1} Munton & Fison spray malt, 20 g l^{-1} Lab M agar No. 2). Pairings between strains were set up by placing 6 mm diameter inoculum plugs (cut from the edges of actively growing 7-d-old colonies) 1 cm apart in the centre of the plates. Stock-cultures were maintained at 4°C on MEA bijou slopes under sterile mineral oil (Fisons: specific gravity 0.86 - 0.89 g ml^{-1}).

Isolation of Homokaryons

Single-basidiospore isolates were obtained from fruit bodies of *H.annosum* collected from *Pinus sylvestris* stumps at Quarry Woods, Box, Wiltshire (GR: ST 833 688) in November 1990. The homokaryons were paired in all possible sib combinations and selected non-sib combinations. Mating and somatic interactions, radial extension rates and morphologies of the isolates were recorded. The P-group status of the isolates was confirmed by pairings with a range of strains from each of three host specialised intersterile races (kindly provided by J.Stenlid). Four mating-type-compatible strains with distinctive growth patterns were selected as 'primary homokaryons' for further study. These included two non-sib-related strains, B1.2 and B6.7, and two sib-related strains from the same fruit body, B3.5 and B3.6.

Production of Heterokaryons

'Secondary heterokaryons' were derived from compatible pairings between the four primary homokaryons. From each pairing, after 4 wk growth at 20°C, small fragments of mycelium were removed from 1 cm on either side of the interaction zone and transferred to 0.02% MEA plates overlaid with sterile cellophane discs. After 4 d at 20°C single hyphal tips were excised according to the method of Butler (1984). Following confirmatory pairings, microscopical examination for clamp connections and molecular analyses (using Jeffreys' fingerprinting probe 33.6 and 33.15 for nuclear genotype confirmation and purified mitochondrial DNA for cytoplasmic genotype confirmation), secondary heterokaryons derived from hyphal tips representing twelve combinations of nuclear and mitochondrial (mt) genotypes were chosen for

further study. These were designated as follows; 1.2/3.5 1.2mt, 1.2/3.5 3.5mt, 1.2/3.6 1.2mt, 1.2/3.6 3.6mt, 1.2/6.7 1.2mt, 1.2/6.7 6.7mt, 3.5/3.6 3.5mt, 3.5/3.6 3.6mt, 3.5/6.7 3.5mt, 3.5/6.7 6.7mt, 3.6/6.7 3.6mt and 3.6/6.7 6.7mt.

Nuclear Number Distributions in Conidia and Spore Germinability

Spore suspensions from each primary homokaryon and derived heterokaryon were prepared by adding 2 ml sterile distilled water to 7 d old cultures whilst the surface of the colony was gently scraped with a sterile Eppendorf tip. The resulting suspension was then diluted to $2x10^5$ spores ml^{-1}. Sub-samples of this suspension were dispensed as 150µl volumes over an area of approximately 150 mm^2 onto the surface of Gelman sterile grid filters (GN6, 47 mm, 0.45 µm) overlaying MEA. Three replicate plates were incubated at 20°C for each of five sampling times; 3, 6, 12, 24 and 48 h. Three randomly chosen plates from each sample were removed from the incubator at each time and their grid filters dried in a desiccator until required for examination.

Grid filters supporting the air dried germinating conidia were mounted on glass slides, flooded with aqueous 4',6-diamidino-2-phenylindole (DAPI: 1 µg ml^{-1}), and examined under x400 magnification using an Olympus BH-2 microscope equipped with a BH-RFL-W epifluorescence unit. Illumination was provided using a 20 UV-W (UG1) excitation filter, BH-DMUV (DM400 + L420) dichroic mirror combination and an L435 barrier filter.

For each replicate preparation two sets of observations were made; firstly the number of germinated and ungerminated conidia in separate fields of view (at least 500 spores were examined) and secondly, the number of nuclei in each of approx. 250 individual ungerminated conidia.

Genotype Recovery from Heterokaryons

Spore suspensions for each heterokaryon were prepared as described above, diluted to 4×10^4 spores ml^{-1} and then spread onto MEA plates in incremental volumes of 50 µl from 50 - 500 µl. After 24 h at 20°C approx. 200 individual well-separated germlings were marked with a sterile dummy objective fitted with a 1 mm diameter cutting tube, transferred to Repli plate wells (Bibby Sterilin Ltd) containing 3 ml MEA and incubated at 20°C for 4 wk. The genotype of each isolate was assigned to one of the original parental homokaryons or to that of

the heterokaryon on the basis of its distinctive cultural characteristics. In preliminary trials, pairings and molecular analyses were performed on a sub-set of the recovered strains to confirm their identity.

Statistical Analyses

Data for nuclear number distributions in ungerminated conidia were analysed using χ^2 tests corrected for discontinuity when appropriate (Milton & Tsokos, 1983). No statistically significant differences were found at the probability level $\alpha = 0.05$ when replicates within any given time period / strain combination were compared - the replicate values were therefore combined during later analysis - see Table 1.1.

The distribution of numbers of conidial nuclei of each isolate for each sampling time was compared with the initial distribution after 3 h. In addition, distributions at each sampling time for each heterokaryon / mitochondrial genotype combination were compared.

Confidence in the validity of these comparisons was provided by preliminary observations over 2 yr indicating that distributions of conidial nuclei remained stable both with respect to the age of spores and to the age of a strain since its isolation.

Calculation of mycelial nuclear ratios and expected recovery rates of homo- and heterokaryotic genotypes via conidia.

A model relating nuclear ratios in a mycelium to the recovery of genotypes via conidia was constructed based on the following assumptions: (1) nuclei are distributed into conidia in the same proportions that they occur in the mycelium ie randomly; (2) there is no interference between nuclei in a heterokaryotic conidium leading to reduced germinability; and (3) no nuclear division occurs within a conidium prior to its germination. On the basis of these assumptions, the proportion R_X of a genotype X in the mycelium, and the expected recovery eHexy, of heterokaryotic conidia from a heterokaryon XY, can be calculated from observed values of the following variables:

P_{t0} = Proportion of conidia with (n) nuclei at time zero

P_{ts} = Proportion of conidia with (n) nuclei at time of sampling

G_{ts} = Total percentage of conidia germinated at time of sampling

Table 1.1. χ^2 *(8 df) to test the hypothesis that no differences in nuclear number distribution occur within three replicates for each incubation time / genotype combination. The table refers to both homokaryons and heterokaryons. P< * = 0.050; nsd = not significantly different*

Homokaryons	3 h	6 h	12 h	24 h	48 h
1.2	5.72	4.58	7.61	5.69	0.88
	nsd	nsd	nsd	nsd	nsd
3.5	8.67	7.76	7.09	14.79	5.16
	nsd	nsd	nsd	nsd	nsd
3.6	7.88	4.79	3.56	2.16	3.04
	nsd	nsd	nsd	nsd	nsd
6.7	8.09	8.98	2.71	4.00	2.66
	nsd	nsd	nsd	nsd	nsd
Heterokaryons					
1.2/3.5 1.2mt	9.58	5.69	4.41	**17.04**	10.91
	nsd	nsd	nsd	*	nsd
1.2/3.5 3.5mt	6.96	1.14	5.04	0.33	1.89
	nsd	nsd	nsd	nsd	nsd
1.2/3.6 1.2mt	6.78	5.58	2.11	4.70	9.58
	nsd	nsd	nsd	nsd	nsd
1.2/3.6 3.6mt	6.02	11.19	3.71	6.99	9.26
	nsd	nsd	nsd	nsd	nsd
1.2/6.7 1.2mt	4.50	1.08	2.41	1.94	2.93
	nsd	nsd	nsd	nsd	nsd
1.2/6.7 6.7mt	3.26	6.26	7.14	2.43	7.14
	nsd	nsd	nsd	nsd	nsd
3.5/3.6 3.5mt	3.24	5.40	4.85	2.77	12.43
	nsd	nsd	nsd	nsd	nsd
3.5/3.6 3.6mt	8.80	3.80	7.54	12.71	3.41
	nsd	nsd	nsd	nsd	nsd
3.5/6.7 3.5mt	2.30	0.83	6.83	4.44	4.66
	nsd	nsd	nsd	nsd	nsd
3.5/6.7 6.7mt	0.81	6.94	5.82	5.60	3.24
	nsd	nsd	nsd	nsd	nsd
3.6/6.7 3.6mt	5.47	3.53	4.51	1.79	0.49
	nsd	nsd	nsd	nsd	nsd
3.6/6.7 6.7mt	12.20	4.80	3.97	5.61	2.50
	nsd	nsd	nsd	nsd	nsd

Hox = Observed recovery of conidia of genotype X

Hoy = Observed recovery of conidia of genotype Y

P(x) = Relative percentage of genotype X conidia to genotype Y conidia as given by equation 1

G_{corr} = Germinability of conidia containing (n) nuclei as given by equation 2

$$P(x) = (Hox/(Hox+Hoy))\ 100 \quad (1)$$

$$G_{corr} = (100-((P_{ts} / P_{t0})(100-G_{ts})))/100 \quad (2)$$

The proportion of a genotype X in a heterokaryotic mycelium can be determined from a standard curve (Figure 1.1), relating hypothetical values of Rx (ranging from 0 to 1) to the expected relative percentage of that genotype, eP(x) calculated from equation 3.

$$eP(x) = (eHox/(eHox+eHoy))\ 100 \quad (3)$$

where:

eHox = Expected recovery of conidia of genotype X, as given by equation 4

eHoy = Expected recovery of conidia of genotype Y, as given by equation 5

$$eHox = \sum_{n=1}^{n=5} R_x^n.P_{t0}.G_{corr} \quad (4)$$

$$eHoy = \sum_{n=1}^{n=5} (1-R_x)^n.P_{t0}.G_{corr} \quad (5)$$

The R_x value for a genotype lies at the point on the standard curve where eP(x) = P(x). The expected recovery of heterokaryotic genotypes from a heterokaryon can be determined from values of Rx using a second standard curve (Figure 1.2) that relates hypothetical values of R_x (from 0 to 1) to calculated values of eHexy - the latter is given by equation 6.

$$eHexy = 1-(eHox+eHoy) \quad (6)$$

Figure 1.1. Hypothetical curves generated from equation 4, for different conidial nuclear number distributions given by the poisson series. Mean values of 1 to 5 nuclei per conidium were used. V_{corr} and G values were set to 100% germination in each case.

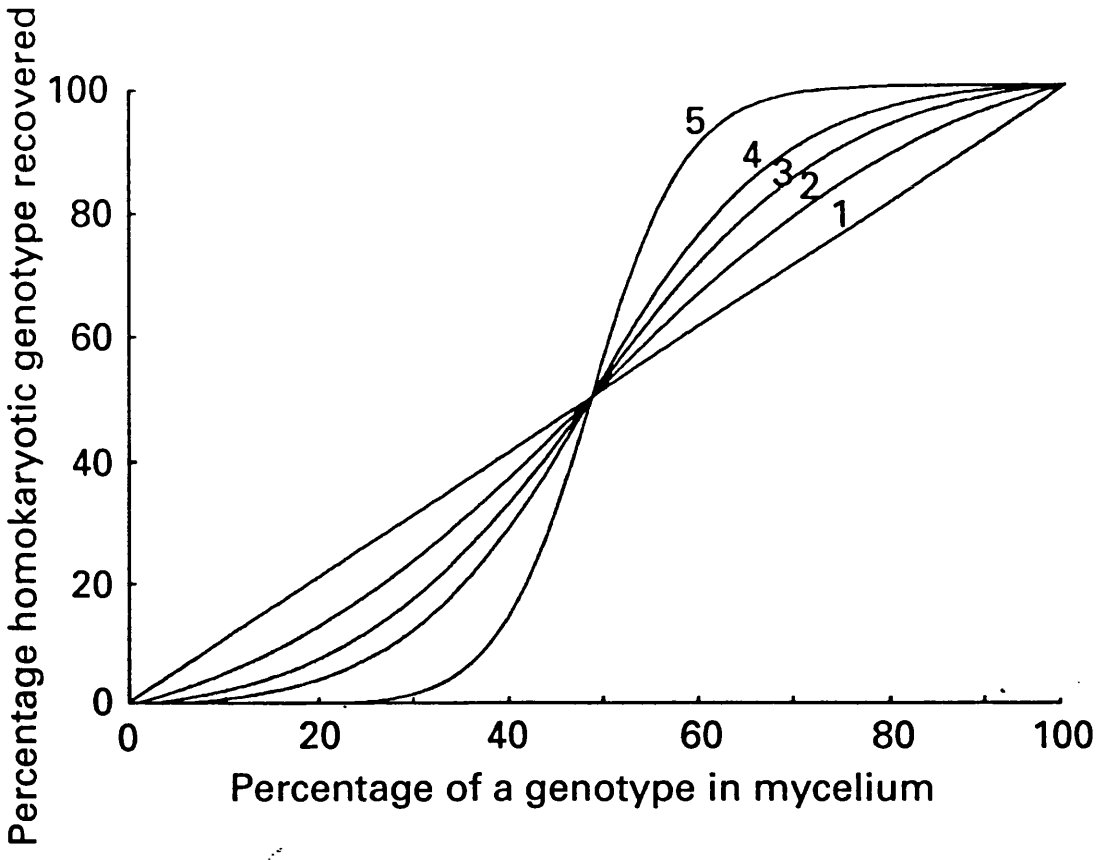
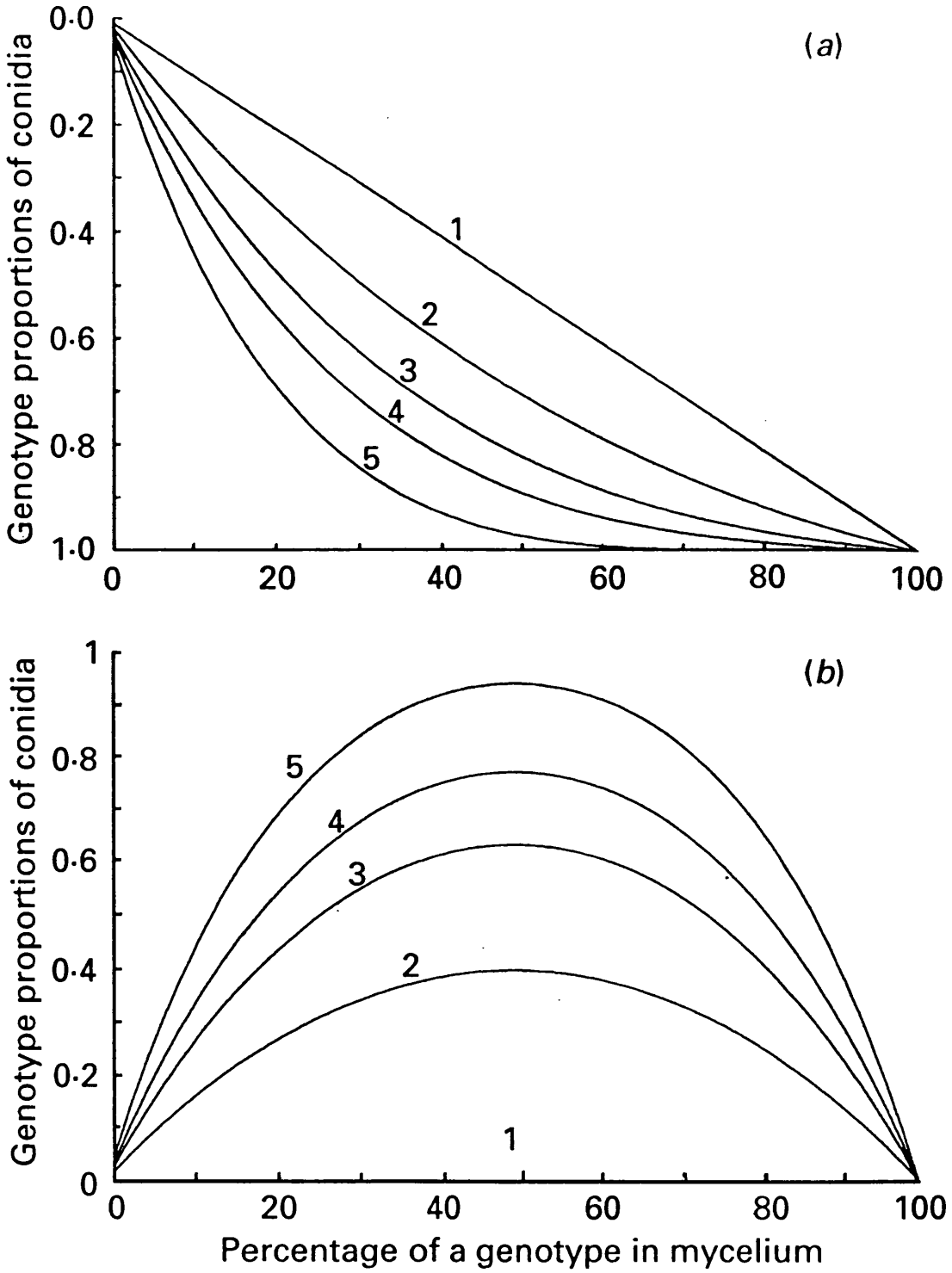


Figure 1.2. Hypothetical curves relating the expected recovery of a) homokaryotic (X) genotype conidia, and b) heterokaryotic (XY) genotype conidia, to the underlying nuclear ratio for different conidial nuclear number distributions given by the poisson series. Mean values of 1 to 5 nuclei per conidium were used. V_{corr} and G values were set to 100% germination in each case.



In this study R_X values were used to assess the consequences of heterokaryotic associations for individual genotypes. The observed recovery of heterokaryotic conidia from mycelia was compared with calculated values, eHexy, using the χ^2 test (1df) in order to ascertain certain aspects of the validity of the model, and to assess the extent to which genomic conflict affects the recovery of heterokaryons.

RESULTS

Interaction patterns and cultural characteristics

Single-basidiospore isolates from fruit bodies of *H.annosum* were morphologically variable, with greater variation between non-sib related than between sib-related strains. Interactions between sib-related strains were consistent with a unifactorial (bipolar) system of mating-type control, as has been found by other workers (Korhonen, 1978, 1987; Chase & Ullrich, 1983; Holt, Gockel & Hütterman, 1983; Stenlid, 1985; Stenlid & Rayner, 1991). The majority of the non-sib pairings (49 out of 52) were mating compatible, resulting in bilateral clamp-connection formation; all of the 'incompatible' non-sib combinations involved a single strain, B6.2.

Self-paired strains merged readily to form an uninterrupted mycelial network. In compatible sib-combinations, secondary mycelia emerged from the interaction interface with no obvious rejection response, whereas incompatible sib-combinations became separated either by a narrow (<2 mm) zone of appressed mycelium, or a wider (>2 mm) zone with very sparse aerial mycelium.

Incompatible non-sib combinations produced wide, flat demarcation zones of strongly appressed, often farinaceous mycelium; this also applied to compatible combinations involving strain B6.7. Compatible combinations generally produced zones of brown pigmentation, only visible from the underside of the plate, accompanied in some pairings by the unilateral or bilateral production of brick-red pseudosclerotial plates.

Characteristics of the four primary homokaryons selected for further study on the basis of the above observations are listed below (mean radial extension rates ± 1SEM (MRER) were determined from 5 replicates grown at 20°C).

Strain B1.2; when initially isolated possessed a slow, dense growth pattern, but upon further sub-culturing switched permanently to a fast-effuse mode. MRER = 0.275±0.006 mm h^{-1}. **Strain B3.5**; a fast-effuse morphology with loose, abundant aerial mycelium. MRER = 0.315±0.013 mm h^{-1}. **Strain B3.6**; a fast-effuse morphology, producing abundant aerial mycelium, possessing a characteristic 'mohair' like appearance. MRER = 0.311±0.009 mm h^{-1}. **Strain B6.7**; a strain producing silky, white mycelium prone to slow-dense / fast effuse switches, generally persisting in the slow-dense mode. Produces prolific quantities of conidia.

MRER = 0.167±0.004 mm h^{-1}. As outlined by Stenlid & Rayner (1989) such variation in morphology between strains of *H.annosum* is not uncommon and may be linked to both environmental and endogenous controls over development.

Genotypic Ratios

Ratios of nuclear genotypes in many heterokaryotic mycelia deviated significantly from 1:1 (Table 1.2). Furthermore, there was a clear dominance hierarchy in the frequency of recovery of homokaryotic conidia from individual heterokaryotic combinations, such that B3.5 > B3.6 > B1.2 > B6.7. There was some indication that the disparity in recovery rates was greatest when strains at the extremes of the hierarchy were associated. Regardless of their position in the hierarchy, homokaryotic genotypes were always more frequently obtained from their resident mycelia than from those that they had invaded. For those associations that produced numbers of heterokaryons statistically significantly different from the expected, fewer than expected were always observed.

Conidial Nuclear Number Distributions

Homokaryotic isolates

Figures 1.3 and 1.4 indicate that homokaryotic isolates produced conidia with a variable number of nuclei (1-5 per spore) as described by Roll-Hansen, (1940); Griffin & Wilson, (1967); Wilson, Miller & Griffin, (1967); Ahrberg, (1975); Hsiang, Edmonds & Driver, (1989) and Stenlid & Häggblom, (1985). The distribution of numbers of nuclei in fresh ungerminated conidia was always unimodal, with the majority of spores being binucleate. No nuclear divisions were observed in any spores prior to the emergence of a germ tube, so the changes in distribution that are to be described cannot have arisen as a result of increases in numbers of nuclei *per se* (this is corroborated by studies of spore germination in other organisms - Punithalingham, 1972; Huang & Patrick, 1974; Stalhammar-Carlemalm,1976; cf Hawes,1978).

In strains B1.2 and B6.7 (Figure 1.4a,d), the binucleate and multinucleate spores germinated more rapidly than the uninucleate conidia, skewing the distribution towards a lower mean, so

Table 1.2. *Recovery of homokaryotic and heterokaryotic conidia from heterokaryons of H.annosum. No. = number of conidia isolated; %A = overall genotype percentage; %B = homokaryotic genotype percentage; χ^2_1 = chi-squared (1df) comparing the observed ratios of homokaryotic genotypes with a 1:1 ratio; %C = calculated mycelial nuclear percentage, %D = expected percentage of heterokaryons, χ^2_2 = chi-squared (1df) comparing the observed and expected recovery of heterokaryons. P< * = 0.050; ** = 0.010; *** = 0.001; nsd = not significantly different.*

Heterokaryon	Conidia	No.	%A	%B	χ^2_1	P<	%C	%D	χ^2_2	P<
1.2/3.5 1.2mt	1.2	52	46	50	0.01	nsd	50	-	-	-
	3.5	51	45	50	-	-	50	-	-	-
	1.2/3.5	12	9	-	-	-	-	23	9.74	***
1.2/3.5 3.5mt	1.2	23	27	32	9.39	***	35	-	-	-
	3.5	49	56	68	-	-	65	-	-	-
	1.2/3.5	15	17	-	-	-	-	21	0.67	nsd
1.2/3.6 1.2mt	1.2	42	35	45	1.06	nsd	46	-	-	-
	3.6	52	43	55	-	-	54	-	-	-
	1.2/3.6	26	22	-	-	-	-	16	2.50	nsd
1.2/3.6 3.6mt	1.2	22	26	35	2.87	*	38	-	-	-
	3.6	41	49	65	-	-	62	-	-	-
	1.2/3.6	21	25	-	-	-	-	19	1.68	nsd
1.2/6.7 1.2mt	1.2	112	54	65	15.7	***	60	-	-	-
	6.7	60	29	35	-	-	40	-	-	-
	1.2/6.7	35	17	-	-	-	-	27	10.7	**
1.2/6.7 6.7mt	1.2	28	39	46	0.41	nsd	48	-	-	-
	6.7	33	46	54	-	-	52	-	-	-
	1.2/6.7	11	15	-	-	-	-	45	25.9	***
3.5/3.6 3.5mt	3.5	41	36	47	0.29	nsd	48	-	-	-
	3.6	46	40	53	-	-	52	-	-	-
	3.5/3.6	27	24	-	-	-	-	54	50.6	***
3.5/3.6 3.6mt	3.5	50	30	60	3.48	*	56	-	-	-
	3.6	33	20	40	-	-	44	-	-	-
	3.5/3.6	86	50	-	-	-	-	51	0.00	nsd
3.5/6.7 3.5mt	3.5	117	67	76	42.9	***	72	-	-	-
	6.7	36	20	24	-	-	28	-	-	-
	3.5/6.7	23	13	-	-	-	-	20	4.99	**
3.5/6.7 6.7mt	3.5	66	48	69	13.5	***	65	-	-	-
	6.7	30	22	31	-	-	35	-	-	-
	3.5/6.7	41	30	-	-	-	-	17	16.2	***
3.6/6.7 3.6mt	3.6	43	41	62	4.19	**	59	-	-	-
	6.7	26	25	38	-	-	41	-	-	-
	3.6/6.7	36	34	-	-	-	-	29	1.74	nsd
3.6/6.7 6.7mt	3.6	85	45	60	5.96	**	57	-	-	-
	6.7	56	30	40	-	-	43	-	-	-
	3.6/6.7	48	25	-	-	-	-	36	9.22	**

Figure 1.3. Conidia from a homokaryotic isolate of *H.annosum* stained with DAPI. Note the presence of uni-, bi- and multi- nucleate cells. Scale bar = 5 μm.

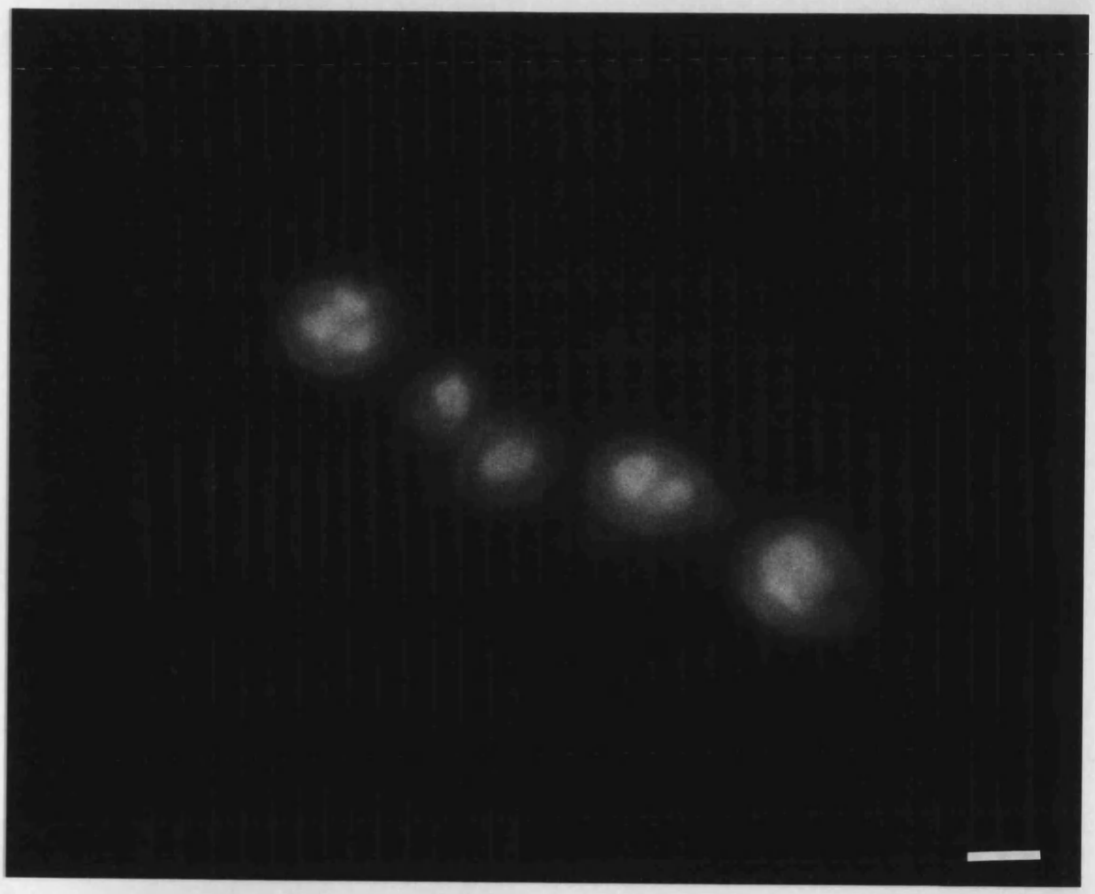
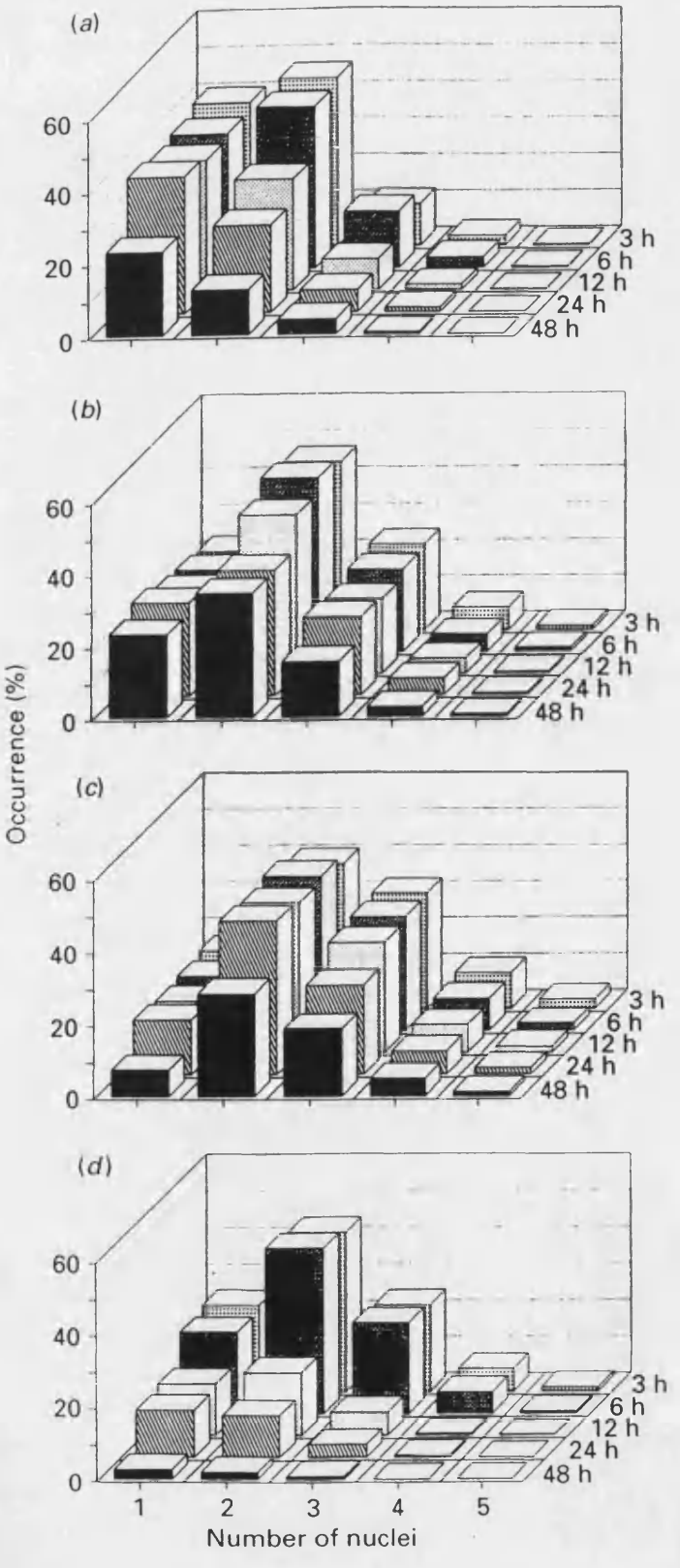


Figure 1.4. Graphs showing nuclear number distributions of ungerminated conidia from homokaryotic isolates of *H.annosum* at different times after plating out onto MEA at 20°C. Values shown are corrected for overall % germination (frequency at any given time x proportion of conidia not germinated). Sample sizes (No. conidia examined) for each time are given below for the 3, 6, 12, 24 and 48 h sampling times respectively. a) Ha 1.2 (809, 872, 834, 812, 815) b) Ha 3.5 (768, 804, 774, 839, 828) c) Ha 3.6 (725, 705, 626, 589, 459) and d) Ha 6.7 (816, 890, 773, 821, 358).



that after 12-24 h the remaining ungerminated conidia were predominantly uninucleate. Statistically, the changes were highly significant (Table 1.3).

Conidial populations of the two sib-related homokaryons, B3.5 and B3.6 (Figure 1.4b,c), remained predominantly binucleate throughout the experiment, although the mean for strain B3.5 did decrease slightly. Whether or not a final uninucleate status would have been attained in either of these two isolates is not clear because conidia from both homokaryons had relatively low levels of overall germination; there may therefore have been insufficient time for any effects of differential germination to emerge. This may prove difficult to test under the current experimental conditions, as germ-tubes and mycelia from germinated conidia obscure the ungerminated conidia after 48 h.

Heterokaryotic isolates

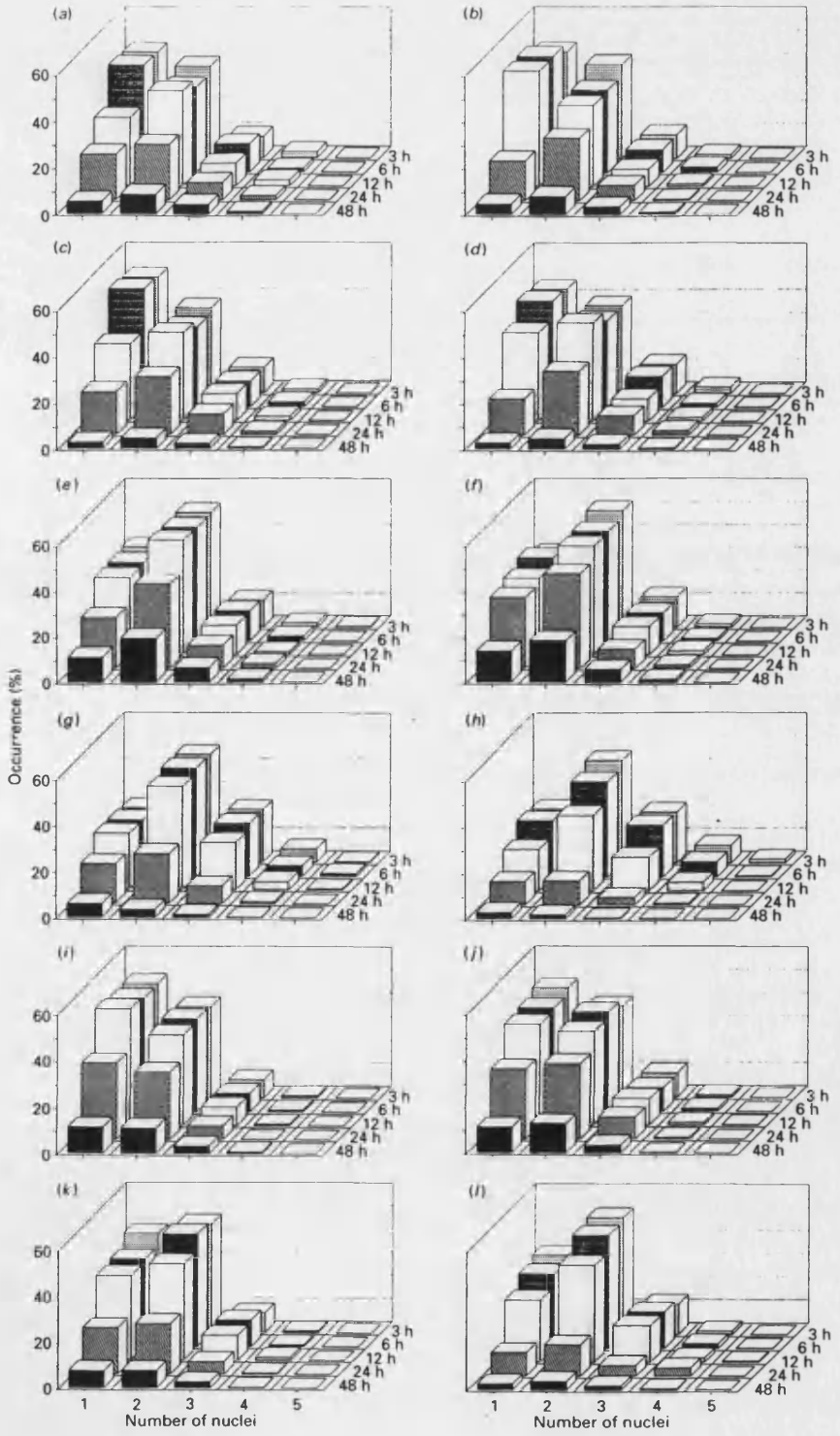
Heterokaryons fell into several categories with respect to the distribution of number of nuclei in conidia and germination patterns (Figure 1.5). Firstly, some strains produced conidia with a distribution of nuclei and germination pattern similar to those of homokaryons. These were 1.2/6.7 1.2mt, 1.2/6.7 6.7mt, 3.5/3.6 3.5mt, 3.5/3.6 3.6mt. In these cases the final distribution was skewed towards a lower mean due to the more rapid germination of multinucleate spores. As with homokaryon B3.5, populations of conidia from 1.2/6.7 1.2mt and 1.2/6.7 6.7mt did not achieve a predominantly uninucleate condition, but as described before this may reflect the low germination rates in these strains.

The remaining strains contrasted with their homokaryotic progenitors in producing conidia whose distribution of nuclei was skewed towards the uninucleate condition. It appears that in these isolates the uninucleate conidia were more viable than their multinucleate counterparts, resulting in a modal value of two for the final distribution for ungerminated spores. The switch from a 'uninucleate' to a 'binucleate' distribution occurred between 12 and 24 h for all but one isolate, 3.5/6.7 3.5mt, which switched later, after 24h.

Table 1.3. χ^2 *(4 df) to test the hypothesis that nuclear number distributions in ungerminated conidia do not vary over time. Comparisons are made for combined replicate data against the initial frequencies at 3h. The table refers to both homokaryons and heterokaryons. P< * = 0.050; ** = 0.010; *** = 0.001; nsd = not significantly different*

Homokaryons	3 h	6 h	12 h	24 h	48 h
1.2	0	6.19	**9.98**	**37.28**	**56.39**
	nsd	nsd	**	***	***
3.5	0	1.97	6.49	**14.40**	**18.21**
	nsd	nsd	nsd	***	***
3.6	0	1.61	2.91	**8.95**	7.51
	nsd	nsd	nsd	*	nsd
6.7	0	1.40	**63.15**	**112.19**	**92.91**
	nsd	nsd	***	***	***
Heterokaryons					
1.2/3.5 1.2mt	0	6.26	**24.15**	**22.47**	**76.21**
	nsd	nsd	***	***	***
1.2/3.5 3.5mt	0	6.89	**10.13**	**31.84**	**68.56**
	nsd	nsd	*	***	***
1.2/3.6 1.2mt	0	2.56	**21.91**	**43.45**	**87.84**
	nsd	nsd	***	***	***
1.2/3.6 3.6mt	0	2.16	**12.20**	**49.31**	**49.85**
	nsd	nsd	**	***	***
1.2/6.7 1.2mt	0	4.82	5.35	**10.02**	**17.68**
	nsd	nsd	nsd	*	***
1.2/6.7 6.7mt	0	2.89	1.27	6.06	8.89
	nsd	nsd	nsd	nsd	nsd
3.5/3.6 3.5mt	0	3.34	3.91	**51.03**	**114.61**
	nsd	nsd	nsd	***	***
3.5/3.6 3.6mt	0	5.49	**14.42**	**84.74**	**118.13**
	nsd	nsd	***	***	***
3.5/6.7 3.5mt	0	0.17	0.18	4.09	**11.35**
	nsd	nsd	nsd	nsd	**
3.5/6.7 6.7mt	0	3.19	3.90	8.34	8.21
	nsd	nsd	nsd	nsd	nsd
3.6/6.7 3.6mt	0	6.08	6.94	5.02	**13.57**
	nsd	nsd	nsd	nsd	***
3.6/6.7 6.7mt	0	3.85	**13.05**	3.83	**33.92**
	nsd	nsd	***	nsd	***

Figure 1.5. Graphs showing nuclear number distributions of ungerminated conidia from heterokaryotic isolates of *H.annosum* at different times after plating out onto MEA at 20°C. Values shown are corrected for overall % germination (frequency at any given time x proportion of conidia not germinated). Sample sizes (No. conidia examined) for each time are given below for the 3, 6, 12, 24 and 48 h sampling times respectively. a) 1.2/3.5 1.2mt (905, 802, 837, 853, 508) b) 1.2/3.5 3.5mt (844, 810, 824, 710, 478) c) 1.2/3.6 1.2mt (830, 814, 833, 733, 403) d) 1.2/3.6 3.6mt (820, 777, 821, 791, 446) e) 1.2/6.7 1.2mt (816, 822, 835, 800, 591) f) 1.2/6.7 6.7mt (783, 692, 838, 746, 672) g) 3.5/3.6 3.5mt (810, 793, 784, 884, 468) h) 3.5/3.6 3.6mt (812, 783, 696, 702, 261) i) 3.5/6.7 3.5mt (800, 790, 731, 701, 548) j) 3.5/6.7 6.7mt (870, 796, 827, 761, 502) k) 3.6/6.7 3.6mt (817, 799, 813, 747, 374) and l) 3.6/6.7 6.7mt (818, 828, 834, 569, 347).



Influence of cytoplasmic background

With the exception of two strains, 3.6/6.7 3.6mt and 3.6/6.7 6.7mt, which showed consistent quantitative differences in germination pattern, cytoplasmic background appeared to have no significant influence over the initial distribution of nuclei or upon the subsequent patterns of germination (Table 1.4). Indeed even in the strains that did show statistically significant differences the overall patterns of germination were similar.

Table 1.4. χ^2 *(4 df) to test the hypothesis that conidial nuclear number distributions do not differ between heterokaryons that contain identical nuclear genotypes but possess different mitochondrial genotypes. The data from three replicates are combined in each comparison. P< * = 0.050; ** = 0.010; *** = 0.001; nsd = not significantly different*

Heterokaryon	3h	6h	12h	24h	48h
1.2/3.5	2.25 nsd	5.17 nsd	**57.49** ***	**46.90** ***	0.83 nsd
1.2/3.6	7.48 nsd	5.28 nsd	4.67 nsd	8.06 nsd	0.89 nsd
1.2/6.7	7.09 nsd	2.59 nsd	0.73 nsd	**11.02** *	5.92 nsd
3.5/3.6	7.05 nsd	7.34 nsd	4.55 nsd	6.24 nsd	6.89 nsd
3.5/6.7	3.80 nsd	5.83 nsd	**11.02** *	**12.38** **	2.63 nsd
3.6/6.7	**19.76** ***	**15.68** ***	**29.68** ***	**11.92** **	**21.85** ***

DISCUSSION

All strains produced conidia containing one to five nuclei except an isolate of *Heterobasidion araucariae*, in which 12% of spores initially contained five or more nuclei. The distribution of numbers of nuclei in conidia produced by any one strain was relatively stable throughout repeated subculturing, and was not affected by the age of the colony from which the spores were taken (Ramsdale, 1991).

In homokaryons predominantly binucleate conidia were produced and uninucleate conidia germinated less readily than others. This could be because conidia with two or more nuclei had a higher 'genomic load', and hence faster rates of synthesis and response to stimuli, as has often been suggested for cells with high ploidy levels (Brodsky & Uryvaeva, 1985).

Heterokaryons that were sib-composed, B3.5 with B3.6, or contained genotypes likely to be closely related, B1.2 with B6.7 (B1.2 and B6.7 have identical mitochondrial DNA RFLP profiles - data not shown), produced conidia with distributions of nuclei and germination patterns reminiscent of homokaryons. However, the remaining non-sib-composed heterokaryons produced predominantly uninucleate conidia which were also more germinable than those with two or more nuclei.

Such differences in distribution of nuclei and germination patterns amongst conidia from heterokaryons, depending on the relatedness of associated nuclear genotypes, may be interpretable in terms of genomic conflict. For all combinations, the allocation of nuclei to conidia and their subsequent patterns of germination may be expected to be directed towards the generation of the highest number of 'fit' progeny. The optimal 'strategy' is to produce those types of conidia that are most vigorous with respect to resource utilisation, show the highest germinability, and which also maintain beneficial combinations of genotypes with a high frequency. For those combinations that are not in conflict, this may be achieved through the production of multinucleate conidia which contain a high proportion of heterokaryotic genotypes and as a result of their high 'genomic load' may be expected to germinate rapidly. Conversely, the production of predominantly uninucleate conidia from associations between conflicting genomes would allow high germinability and promote the escape of a genotype.

Not only the rates of germination but also the rates of recovery of particular genotypes from a heterokaryon may be influenced by the distribution of nuclei and genomic conflict. Examination of Figure 1.2 shows that for any given underlying mycelial nuclear ratio, the probability of recovery of a homokaryotic genotype decreases non-linearly as the mean nuclear number per spore increases, whereas the numbers of heterokaryons recovered increase. Moreover, differential spore germinability (taken into account by the model) influences the recovery of genotypes in effect by modifying the distribution of number of nuclei in conidia.

The calculated underlying nuclear ratios frequently departed from 1:1. Indeed there appeared to be a strict dominance hierarchy, in which one genotype always outnumbered its partner. The occurrence of asymmetric ratios within an individual mycelium has implications for the biology of the organism at many levels of organisation, not least in terms of an increased potential for developmental plasticity, as well as direct effects on the frequency of a genotype in a natural population. It is possible to speculate, as others have done (eg. Beadle & Coonradt, 1944; Jinks, 1952; Pittenger, Kimball & Atwood, 1953; Davis, 1960a,b; Sidhu, 1983a,b), that mycelia respond to a range of environmental stimuli by altering their nuclear ratios in an adaptive fashion.

The observed genomic 'dominance hierarchy' is reminiscent of those described for *Penicillium*, *Fusarium*, and *Schizophyllum* (Jinks, 1952; Sidhu, 1983a,b and Raper, 1985). In these organisms a clear correlation has been demonstrated between the position of a genotype in the hierarchy and the growth rates of the parental genotypes. The same correlation applies to the strains of *H.annosum* we have examined. In *Schizophyllum* the hierarchy was linked directly to the alleles possessed by the associated genomes at the B-mating-type locus (Raper, 1985). Taken together these observations imply that there may be a common factor, or combination of factors that determines the frequency of nuclear genotypes in a mycelium. Whatever the mechanisms that determine the final stable nuclear ratios, there must always be an initial convergence of the rates of replication of the associated genomes if monopolization or instability are to be avoided. The regulatory pathways involved may therefore include components that influence key cell cycle decision points - perhaps in a manner analogous to the

synchronization of cell cycle events that precede mating in *Saccharomyces cerevisiae* (Forsburg & Nurse, 1991).

Regardless of position in the dominance hierarchy, homokaryotic genotypes were more frequently isolated from their 'resident' cytoplasmic backgrounds than from those that they had invaded. There may be several reasons for this. Firstly, a nuclear genome may be 'co-adapted' to its resident cytoplasm and so may co-exist more stably with its natural cytoplasmic partners than will an 'invading' genome (Eberhard, 1980; De Francessco, Attardi & Croce, 1980; Cosmides & Tooby, 1981; Avise, 1991; Bonnema *et al.*, 1992; Wolters, Koorneet & Gilissen, 1993). Secondly, genomes may fare better in their resident mycelia because of their numerical dominance at the outset of an interaction. Thirdly, dominance of a genotype could reflect differential spore viabilities for specific nucleo-cytoplasmic combinations. However, no clear cytoplasmic effects upon spore germination were seen in this study. Additional work, examining the outcomes of pairings between homokaryotic strains re-isolated from 'non-native' cytoplasmic backgrounds may distinguish between these possibilities (see later).

Genotype non-equivalence or ratio asymmetry has been described in several ascomycete fungi (eg Pontecorvo, 1946; Jinks, 1952; Raper & Fennell, 1953). However, its importance has been neglected after the realization that systems of heterokaryon incompatibility in the majority of natural populations of ascomycetes restrict somatic heterokaryosis to clonal or near clonal sub-populations (Croft & Jinks, 1977). Limited somatic heterokaryosis has been observed in some outcrossing ascomycetes, though there appears to be a general lack of stability in these systems (Sharland & Rayner, 1986, 1989a). Studies of ratio asymmetry in basidiomycetes have largely involved either the recovery of genotypes from mycelia under artificial / strongly selective conditions (eg Miles & Raper, 1956; Kerruish & DaCosta, 1963) or, from tightly controlled dikaryotic fungi (Aschan, 1954; Snider & Raper, 1965; Leonard, Garber & Dick, 1978; Raper, 1985). Also, these studies have mostly employed the use of forced heterokaryons between auxotrophic mutants, and so their relevance to natural populations is doubtful (Caten & Jinks, 1966).

An examination of ten heterokaryotic field isolates indicated that seven produced conidia that were predominantly binucleate - as with homokaryons (see later section). This suggests that

selection may operate in natural populations against conflicting combinations of genetic partners.

Further work will examine patterns of nuclear behaviour produced by heterokaryons generated from pairings between allopatrically derived parental homokaryons. Such combinations may be expected to lead to greater genomic conflict. Indeed genomic conflict may even be responsible for the intersterility phenomena observed in *H.annosum* (Korhonen, 1978; Chase & Ullrich, 1988; Chase *et al.*, 1989; Capretti *et al.*, 1990; Stenlid & Karlsson, 1991).

SECTION II

EFFECT OF TEMPERATURE ON GENOTYPE RATIOS WITHIN HETEROKARYOTIC MYCELIA OF *HETEROBASIDION ANNOSUM* (FR.) BREF.

INTRODUCTION

The existence of a strict dominance hierarchy affecting nuclear ratios within heterokaryotic mycelia of *Heterobasidion annosum*,, where one nucleus high in the series 'outcompetes' any with a lower ranking, suggests that intrinsic differences between the nuclei are responsible for their competitive abilities. In *Schizophyllum commune*, control over such a hierarchy was linked directly to the alleles possessed by the competing nuclei at their B-mating-type factors (Raper, 1985). This implied therefore that the outcome of nuclear competition within a heterokaryon could be entirely predicted from a knowledge of the alleles that the competitors possess.

A clear correlation has also been established in a number of fungi between the position of a genotype in a competitive hierarchy and its growth rate as a homokaryon under the particular conditions at which the nuclear ratios are assessed (Jinks, 1952; Sidhu, 1983a,b; Raper, 1985; Typas & Heale, 1976; Wang, 1980). A tentative relationship of this nature was also noted earlier from the patterns of genotype recovery from laboratory-synthesized sympatric heterokaryons of *Heterobasidion annosum.*

Such correlations have been used by some (Jinks 1952; Sidhu 1983a,b; Davis, 1966) to implicate selection as the primary determinant of nuclear ratios within heterokaryotic mycelia. Many cases have however been reported where the role of selection is ambiguous, or contrasts with the outcomes that would be expected under an imposed selection regime.

The results reported earlier in sympatric heterokaryons of *H.annosum* also revealed that irrespective of their position within the interaction hierarchy, homokaryons were always more frequently isolated from their resident 'maternal' mycelia than the mycelia that they had invaded. Similar behaviour was reported in *Rhizoctonia solani* by Anderson *et al* (1972). Furthermore, prior to stabilization, heterokaryons behaved more like homokaryons; a fact that was attributed to the initial bias towards maternal nuclei.

The general notion of a stable nuclear ratio has been put into question by a number of studies. For example, Wang (1980) found that the frequencies of auxotrophic mutants within *Agaricus bisporus* were subject to directional changes, whilst nuclear ratios within common-A

heterokaryons of *Schizophylllum commune* fluctuated over time (Snider & Raper, 1958; Wessels *et al.*, 1976; cf Raper, 1985).

Taken together, these observations imply that both initial conditions (genetic inputs) and imposed selection pressures might influence the ratios observed at any given point in time. The following study addresses such issues by examining the establishment and stability of nuclear ratios under a variety of temperature regimes within sympatric heterokaryons of *Heterobasidion annosum* .

MATERIALS AND METHODS

Strains, pairings and sub-culturing

Unless otherwise stated, all strains were grown in 90 mm diameter unvented Petri dishes containing approximately 15 ml MEA. Stock cultures were maintained on MEA bijou slopes under sterile mineral oil (Fisons: specific gravity 0.86-0.89 g ml^{-1}) at 4°C and without oil at the experimental temperatures of 10,15,20 and 25°C.

Six heterokaryons of known nuclear and mitochondrial genotype generated at 20°C prior to the start of these experiments, were sub-cultured onto MEA plates at 10,15,20 and 25°C. The strains were maintained and sub-cultured at their experimental temperatures for the duration of the investigation. An 8 wk period of acclimatization was imposed prior to the examination of the strains.

During this acclimation period, homokaryon B1·2 was paired with strains B3·5, B3·6 and B6·7 at 10,15,20 and 25°C to generate 'repeats' of the original heterokaryotic strains. Pairings between strains were set up by placing 6 mm diameter inoculum plugs (cut from the edge of actively growing colonies maintained at the appropriate temperature for 4 wk) 1 cm apart in the centre of MEA plates. After four weeks growth, regardless of temperature regime, small sub-cultures were taken from 1 cm on either side of the interaction zone. Single hyphal-tips were excised from these cultures according to the method of Butler (1984). Strain designations for the original heterokaryons and their 'repeat' equivalents are given in Table 2.1.

Nuclear numbers in conidia and spore germinability

Conidial suspensions were prepared from heterokaryotic mycelia as described previously. Nuclear numbers in two replicates of a minimum of 250 ungerminated conidia obtained from 4 wk old heterokaryons were assessed at 3 h and 24 h following initial transfer of conidia to sterile grid-filters overlying MEA. Nuclei were visualized by staining air-dried spores DAPI (1 μg ml^{-1}). Germinability was assessed by counting the number of germinated and ungerminated conidia in random fields of view under the microscope ($\chi^2$00 magnification) until at least 500 spores had been examined.

Table 2.1. *Designation of Heterobasidion annosum strains examined*

Strain code	Parents	Temperatures
O 1·2/3·5 1·2mt *	1·2 + 3·5	20°C _ 10,15,20,25°C
O 1·2/3·5 3·5mt *	1·2 + 3·5	20°C _ 10,15,20,25°C
O 1·2/3·6 1·2mt *	1·2 + 3·6	20°C _ 10,15,20,25°C
O 1·2/3·6 3·6mt *	1·2 + 3·6	20°C _ 10,15,20,25°C
O 1·2/6·7 1·2mt *	1·2 + 6·7	20°C _ 10,15,20,25°C
O 1·2/6·7 6·7mt *	1·2 + 6·7	20°C _ 10,15,20,25°C
R 1·2/3·5 1·2mt *	1·2 + 3·5	10°C _ 10°C:25°C->25°C
R 1·2/3·5 3·5mt *	1·2 + 3·5	10°C _ 10°C:25°C->25°C
R 1·2/3·6 1·2mt *	1·2 + 3·6	10°C _ 10°C:25°C->25°C
R 1·2/3·6 3·6mt *	1·2 + 3·6	10°C _ 10°C:25°C->25°C
R 1·2/6·7 1·2mt *	1·2 + 6·7	10°C _ 10°C:25°C->25°C
R 1·2/6·7 6·7mt *	1·2 + 6·7	10°C _ 10°C:25°C->25°C

* with appropriate temperature designation

Strain code	Parents
S **1·2**/6·7 3·5mt	1·2N 3·5mt :1·2/3·5 + 6·7N 3·5mt :3·5/6·7
S 1·2/**6·7** 3·5mt	1·2N 3·5mt :1·2/3·5 + 6·7N 3·5mt :3·5/6·7
S **1·2**/6·7 6·7mt	1·2N 6·7mt :1·2/6·7 + 6·7N 1·2mt :1·2/6·7
S 1·2/**6·7** 1·2mt	1·2N 6·7mt :1·2/6·7 + 6·7N 1·2mt :1·2/6·7

The strains were maintained at 20°C.

Genotype recovery from heterokaryons

Preliminary experiments comparing germination rates of spores taken from specific incubation regimes and then plated out at that temperature, or at 20°C, indicated that after 24 h incubation, high germination rates were obtained at 20°C. Consequently, 20°C was chosen as the most appropriate temperature for the recovery of genotypes from a mycelium in the following experiments.

Spore suspensions from each heterokaryon were prepared at a variety of dilutions and were spread onto MEA plates. After 24 h incubation approximately 200 individual well-separated germlings were marked with a dummy objective fitted with a 1 mm diameter cutting tube, and transferred to separate Repli plate wells (Bibby Sterilin Ltd) each containing 3 ml MEA. Plates were incubated at 20°C for 4 wk and then the genotypes of the colonies were assigned on the basis of their cultural characteristics. The number of germlings that failed to develop were recorded as an indication of the level of post-germination mortality (PGM).

Additional genotype recovery experiments

In an experiment to distinguish between initial numerical dominance and cytoplasmic background as determinants of genotype ratio asymmetry within a mycelium, homokaryons derived from heterokaryons via single conidia were 'back-crossed' to generate a second series of heterokaryons with 'native' and 'foreign' cytoplasmic background - see Table 2.1. These strains were then subjected to genotype recovery analysis as described above.

Growth rate estimation of parental and 'derived' homokaryons

For each of the four parental homokaryotic strain / temperature combinations, and for all possible types of homokaryons derived via conidia from the experimental heterokaryons, five MEA plates were centrally inoculated with 6 mm diameter plugs cut from the margin of actively growing mycelia. Plates were then incubated in darkness, at the appropriate experimental temperatures. Colony margin positions were marked onto the underside of the plates at daily intervals from 4-12 days following inoculation. Growth rates were estimated by measuring the diameter of colonies across two perpendicular axes (passing through the centre of the colony) after 4 d and 7 d (25,20°C); 4 d and 8 d (15°C) and 7 d and 11 d (10°).

Statistical analyses

Data for nuclear number distributions in ungerminated conidia were analysed using χ^2 tests corrected for discontinuity when appropriate (Milton & Tsokos, 1983). Consistency of results was confirmed, so replicate data were combined during later analysis.

The distribution of nuclei in ungerminated conidia within a strain at the 24 h sampling time was compared to that observed after 3 h. Comparisons were also made between strains with respect to temperature, conditions of heterokaryon formation and cytoplasmic background. Ratios of nuclear genotypes within heterokaryotic mycelia were calculated using the equations described previously. Genotype ratios within each heterokaryon were compared with a 1:1 ratio, with equivalent heterokaryons generated under different conditions, and with heterokaryons of identical nuclear, but different cytoplasmic compositions. The recovery of heterokaryotic conidia was compared to that expected from the model.

The level of post-germination mortality shown by each strain at each temperature was compared to that shown by the parental homokaryons when recovered directly from homokaryotic mycelia. The relationship between post-germination mortality and the recovery of specific genotypes was assessed used a combination of regression and correlation analyses.

RESULTS

Effects of temperature and temperature change on growth

Transfer of heterokaryons generated at 20°C, and 'parental' homokaryons to a range of incubation temperature regimes resulted in changes in colony growth pattern (Figure 2.1,2.2), which were particularly evident at lower temperatures. Radial growth rates of colonies at 10° and 15°C were considerably lower than those at 20°C or, 25°C (Table 2.2). The growth rates were however higher than they might have been, had the colonies maintained the same mycelial geometry exhibited at more elevated temperatures. Initial growth was very sparse at 10° and 15°C, but more polarized than at higher temperatures since hyphae branched less frequently and at more acute angles. As a result of this polarity, isolated hyphal-tips often developed into sectors rather than circular colonies. Colonies at 15° and 20°C were generally similar in appearance after 4 wk growth, but those at 15°C developed at a considerably slower rate. Following limited exposure to light, heterokaryotic strains produced pseudosclerotial plates regularly at 20°C, but only occasionally at 15°C. The onset of PSP production was slower at 15°C. Strains maintained at 10° or 25°C were never observed to produce PSPs. Heterokaryotic strains grown at 25°C were prone to phenotypic switching and to senescent growth.

Relative growth rates of the parental homokaryotic strains altered at different temperatures; at 10° and 15°C (3.5 > 3.6 > 1.2 > 6.7), at 20°C (3.6 > 3.5 > 1.2 > 6.7) and at 25°C (3.5 > 1.2 > 3.6 > 6.7) - see Table 2.2. The growth rates of homokaryons derived from heterokaryons were variable, with no consistent pattern of variation. In some cases the growth rates of the derived homokaryons were very much faster or slower than either of their parental homokaryons.

Temperature had no visible effect upon the final outcome of interactions between primary parental homokaryons. Overall, patterns of heterokaryon emergence were similar to those described previously. However the rates at which equivalent stages in the progression of an interaction were attained were reduced at lower temperatures. The changes in the relative growth rates of the parental homokaryons also altered the final size of the domain occupied by reciprocal heterokaryons on culture plates - see Figure 2.3.

Figure 2.1. Heterokaryons of *H.annosum* generated at 20°C after prolonged sub-culturing at 25, 20, 15 and 10°C. (a) 1.2/3.5 1.2mt; (b) 1.2/3.5 3.5mt; (c) 1.2/3.6 1.2mt; (d) 1.2/3.6 3.6mt; (e) 1.2/6.7 1.2mt; (f) 1.2/6.7 6.7mt. All plates represent 20 d growth in darkness.

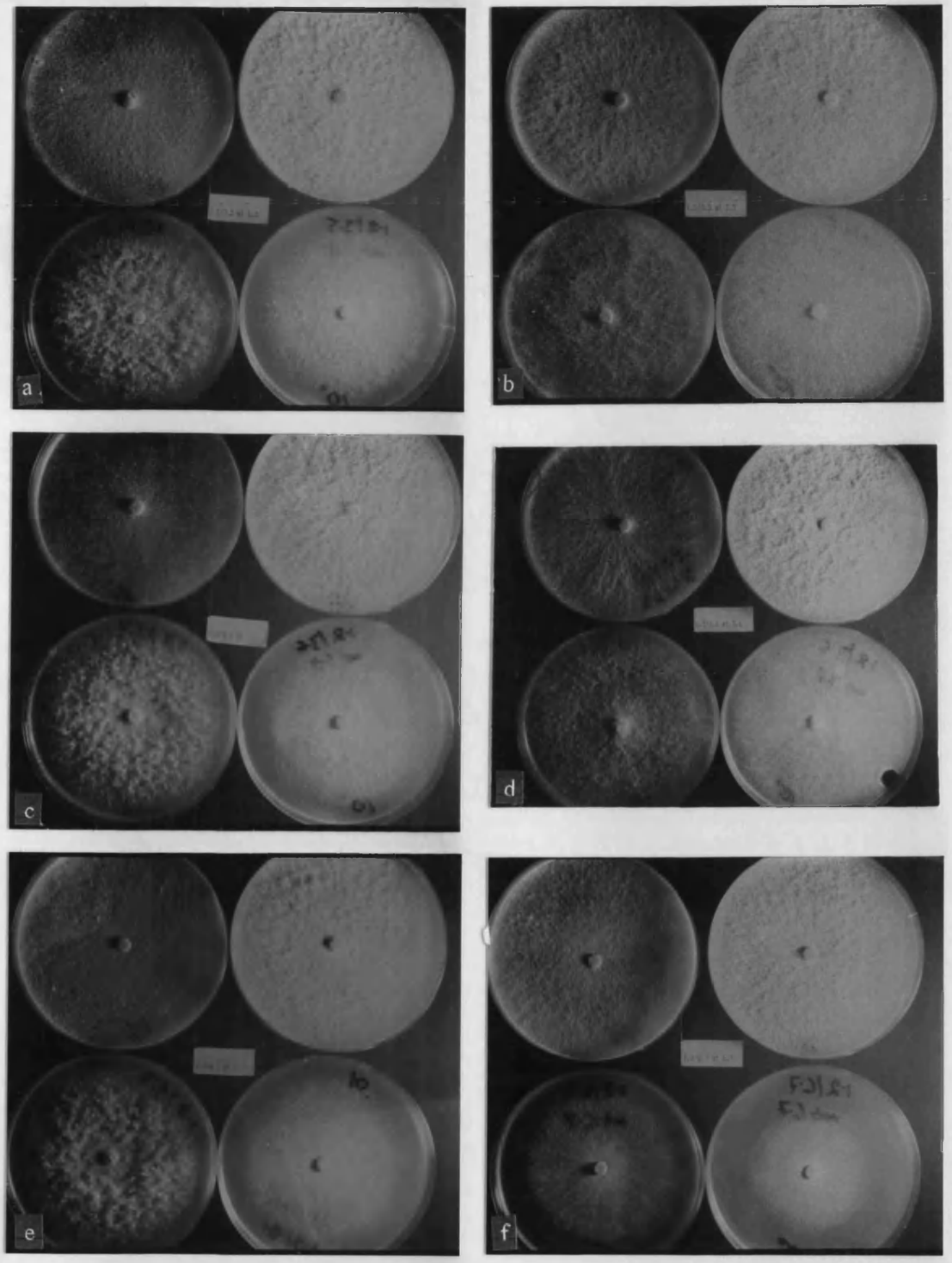


Figure 2.2. Phenotypic variation in a genotypically identical heterokaryotic strain generated at different temperatures. Plates grown in darkness for 14 days.

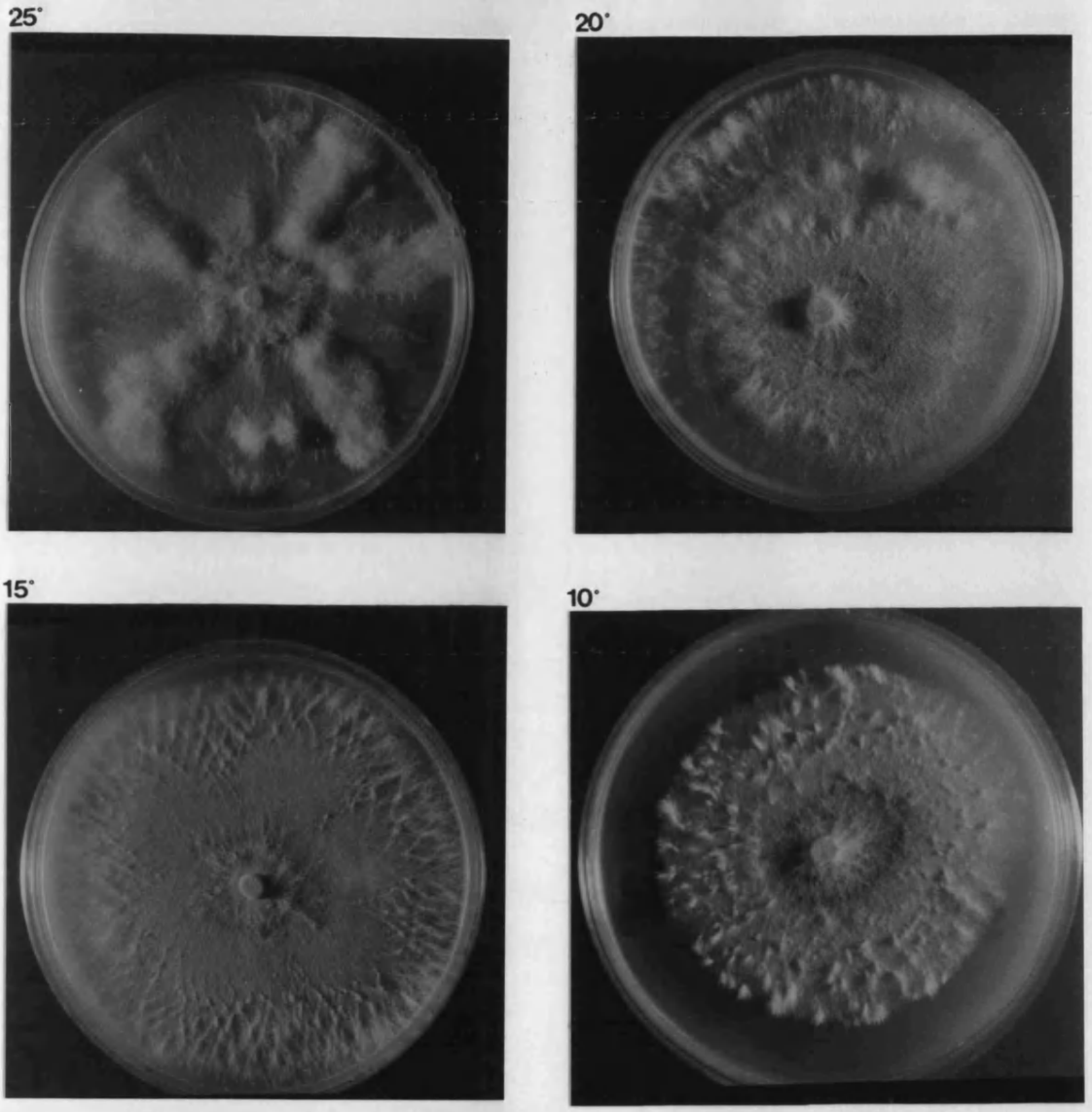


Table 2.2. *Mean radial extension rates (mm d^{-1} ± 1 SEM) for parental and 'derived' homokaryons at different temperatures.*

Strain	25°C	20°C	15°C	10°C
1·2N	0.339± C	0.335± D	0.113± D	0.118± E
	0.007	0.006	0.001	0.001±
1·2N 1·2mt	0.011± A	0.011± A	0.031± B	0.010± A
:1·2/3·5	0.005	0.002	0.002	0.003
1·2N 1·2mt	0.338± C	0.315± C	0.089± D	0.102± D
:1·2/3·6	0.006	0.005	0.006	0.003
1·2N 1·2mt	0.229± B	0.200± B	0.085± C	0.081± C
:1·2/6·7	0.017	0.003	0.002	0.003
1·2N 3·5mt	0.424± D	0.383± E	0.149± F	0.149± F
:1·2/3·5	0.008	0.010	0.003	0.004
1·2N 3·6mt	0.363± CD	0.396± F	0.134± E	0.166± G
:1·2/3·6	0.053	0.005	0.003	0.004
1·2N 6·7mt	0.031± A	0.015± A	0.009± A	0.017± B
:1·2/6·7	0.007	0.004	0.002	0.003
F-ratio	55.21	404.9	233.6	688.8
P<	***	***	***	***
3·5N	0.387± CD	0.342± E	0.152± BC	0.152± B
	0.003	0.006	0.003	0.004
3·5N 3·5mt	0.054± A	0.032± A	0.012± A	0.004± A
:1·2/3·5	0.003	0.003	0.001	0.001
3·5N 3·5mt	0.407± D	0.390± F	0.164± C	0.154± B
:3·5/3·6	0.007	0.007	0.002	0.010
3·5N 3·5mt	0.328± BC	0.368± CD	0.141± B	0.148± B
:3·5/6·7	0.026	0.007	0.003	0.017
3·5N 1·2mt	0.339± BC	0.352± D	0.140± B	0.173± D
:1·2/3·5	0.006	0.002	0.004	0.003
3·5N 3·6mt	0.328± B	0.335± BC	0.158± C	0.153± B
:3·5/3·6	0.010	0.005	0.002	0.007
3·5N 6·7mt	0.326± B	0.324± B	0.149± BC	0.163± C
:3·5/6·7	0.007	0.004	0.004	0.005
F-ratio	73.69	862.1	106.6	880.6
P<	***	***	***	***

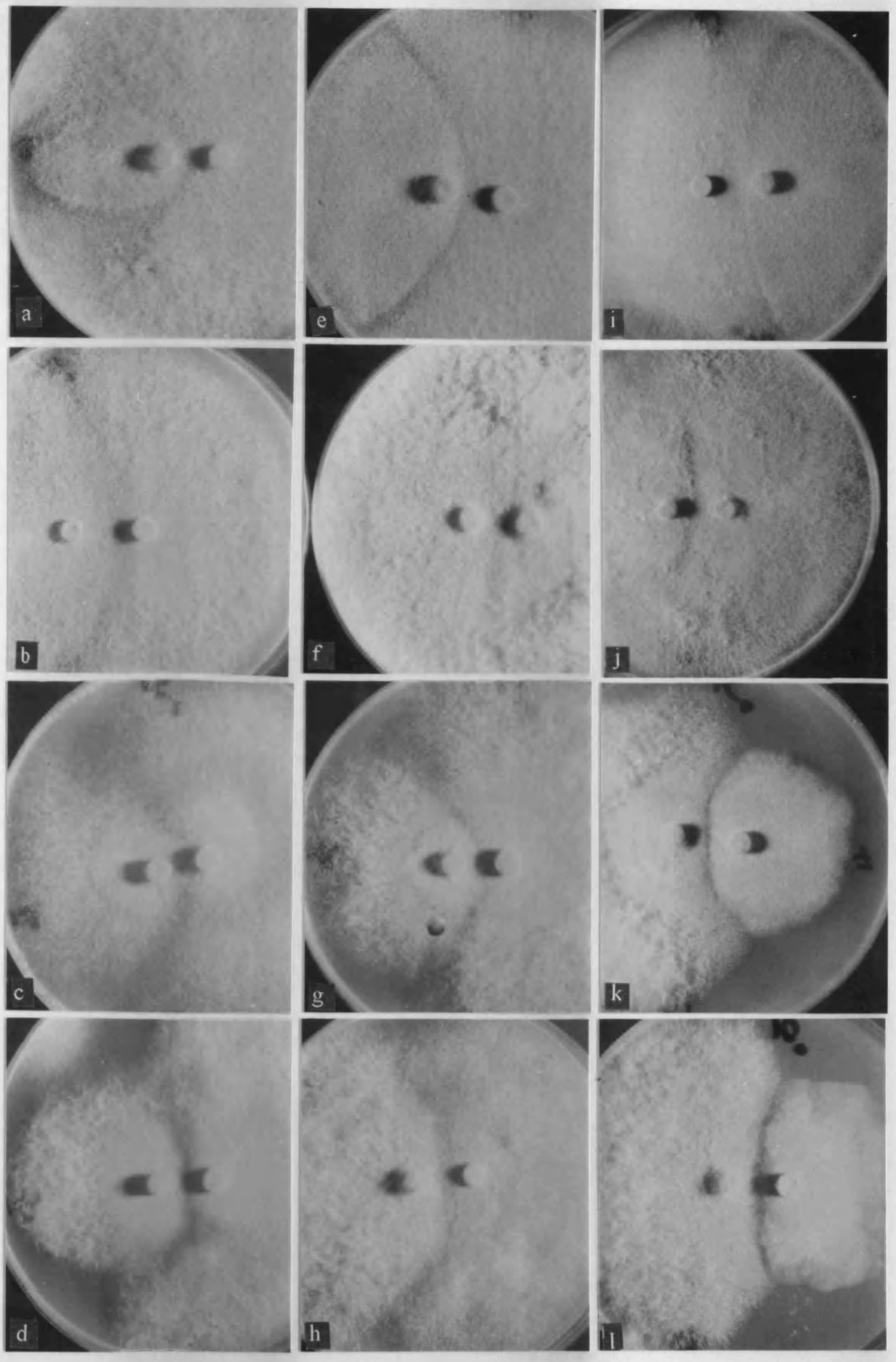
Variance ratio, F (6,28 df), indicates variation amongst the 7 genotype treatments (critical point = 5.39); P < ***, 0.001. Growth rates of genotypes within a temperature do not differ significantly from one another at the P < 0.050 level according to Duncan's multiple range test grouping if the letters are identical.

Table 2.2 continued. *Mean radial extension rates (mm d^{-1} ± 1 SEM) for parental and 'derived' homokaryons at different temperatures.*

Strain	25°C	20°C	15°C	10°C
3·6N	0.326± CD	0.351± D	0.147± E	0.148± E
	0.005	0.008	0.002	0.004
3·6N 3·6mt	0.349± D	0.410± E	0.133± DE	0.098± C
:1·2/3·6	0.013	0.006	0.044	0.007
3·6N 3·6mt	0.257± BC	0.191± C	0.105± C	0.157± E
:3·5/3·6	0.054	0.027	0.006	0.004
3·6N 3·6mt	0.011± A	0.041± A	0.018± A	0.020± A
:3·6/6·7	0.003	0.007	0.018	0.020
3·6N 1·2mt	0.319± C	0.361± D	0.121± DC	0.133± D
:1·2/3·6	0.006	0.009	0.002	0.003
3·6N 3·5mt	0.189± B	0.096± B	0.068± B	0.066± B
:3·5/3·6	0.033	0.024	0.007	0.006
3·6N 6·7mt	0.042± A	0.231± C	0.049± B	0.064± B
:3·6/6·7	0.008	0.005	0.008	0.004
F-ratio	29.41	73.26	52.76	99.94
P<	***	***	***	***
67N	0.075± C	0.034± A	0.012± A	0.011± A
	0.011	0.002	0.001	0.003
6·7N 6·7mt	0.034± A	0.029± A	0.011± A	0.013± A
:1·2/6·7	0.003	0.007	0.001	0.002
6·7N 6·7mt	0.068± BC	0.089± B	0.067± C	0.051± C
:3·5/6·7	0.005	0.007	0.008	0.004
6·7N 6·7mt	0.023± A	0.026± A	0.010± A	0.016± A
:3·6/6·7	0.002	0.004	0.002	0.002
6·7N 1·2mt	0.318± E	0.218± C	0.049± B	0.031± B
:1·2/6·7	0.009	0.027	0.004	0.007
6·7N 3·5mt	0.245± D	0.193± C	0.075± C	0.077± D
:3·5/6·7	0.011	0.006	0.006	0.002
6·7N 3·6mt	0.048± AB	0.026± A	0.012± A	0.017± A
:3·6/6·7	0.003	0.002	0.002	0.004
F-ratio	204.4	55.36	42.41	49.49
P<	***	***	***	***

Variance ratio, F (6,28 df), indicates variation amongst the 7 genotype treatments (critical point = 5.39); P < ***, 0.001. Growth rates of genotypes within a temperature do not differ significantly from one another at the P < 0.050 level according to Duncan's multiple range test grouping if the letters are identical.

Figure 2.3. Outcome of interactions between strains carried out at different temperatures: 1.2 x 3.5 at (a) 25°C, (b) 20°C, (c) 15°C, (d) 10°C; 1.2 x 3.6 (e) 25°C, (f) 20°C, (g) 15°C, (h) 10°C; 1.2 x 3.6 at (i) 25°C, (j) 20°C, (k) 15°C and (l) 10°C. All plates incubated for 21 d in darkness.



Nuclear numbers in conidia

The allocations of nuclei to conidia derived from heterokaryons of *H.annosum*, generated or subcultured under different temperature regimes, are presented in Figures 2.4-2.7. Replicate measurements of the distribution of nuclei to conidia at different temperatures for the 'original' strains show little variability (Table 2.3a), although some statistically significant differences between replicates were detected for strains at 25° and 15°C ($P < 0.05$). For the 'repeat' heterokaryons, replicate variability was high in some strains at 15° and 10°C, and low at 20° and 25°C (Table 2.3b).

The majority of strains produced conidia that were predominantly binucleate at low temperatures (10° and 15°C). At 10°C the mean number of nuclei per conidium decreased in all cases after 24 h incubation, suggesting more rapid germination of multinucleate spores. Conidia from the 'repeat' strains, and the 'original' strains O1.2/6.7 1.2mt and O1.2/6.7 6.7mt, when taken from mycelia at 15°C germinated in a similar fashion to conidia at 10°C. The remaining 'original' strains at 15°C behaved differently; numbers of nuclei within conidia shifted to give a higher mean after 24 h, indicating that both binucleate and uninucleate conidia germinated more rapidly than multinucleate conidia. At the high temperatures (20° and 25°C), conidia from the 'original' and 'repeat' strains of 1.2/6.7 were mostly binucleate. In these strains, either all conidia were equally germinable, or the binucleate conidia germinated more readily. This pattern was also observed for the 'repeat' R1.2/3.5 strains. For the residual strains, the majority of conidia were either uninucleate or binucleate. Conidia with the predominant nuclear status were more germinable than their multinucleate counterparts, increasing the mean numbers of nuclei per spore over time. Tables 2.4a and 2.4b examine the significance of these changes.

Clear differences were detected when 'original' and 'repeat' strains are compared at any given temperature (Table 2.5). Differences were also detected when nuclear allocation patterns for any one strain were compared under a range of temperatures (Table 2.6). These differences were highly significant ($P<0.001$ in many comparisons). An examination of the chi-squared values themselves, reveals that levels of variability were consistently higher amongst the 'repeat' strains than amongst the 'original' strains.

Figure 2.4. Distribution of numbers of nuclei in ungerminated conidia from 'original' and 'repeat' heterokaryotic isolates of *H.annosum* at 3 h and 24 h after plating out onto MEA at 25°C. Values shown are corrected for overall percentage germination (frequency at any given time x proportion of conidia not germinated). Sample sizes (number of conidia examined) for the 3 and 24 h sampling times were, respectively: (a) O1·2/3·5 1·2mt 25°C, (563, 206); (b) O1·2/3·5 3·5mt 25°C, (562, 155); (c) R1·2/3·5 1·2mt 25°C, (432, 351); (d) R1·2/3·5 3·5mt 25°C, (440, 403); (e) O1·2/3·6 1·2mt 25°C, (498, 561); (f) O1·2/3·6 3·6mt 25°C, (544, 459); (g) R1·2/3·6 1·2mt 25°C, (495, 519); (h) R1·2/3·6 3·6mt 25°C, (432, 560); (i) O1·2/6·7 1·2mt 25°C, (502, 826); (j) O1·2/6·7 6·7mt 25°C, (489, 153); (k) R1·2/6·7 1·2mt 25°C, (344, 430) and (l) R1·2/6·7 6·7mt 25°C, (466, 432).

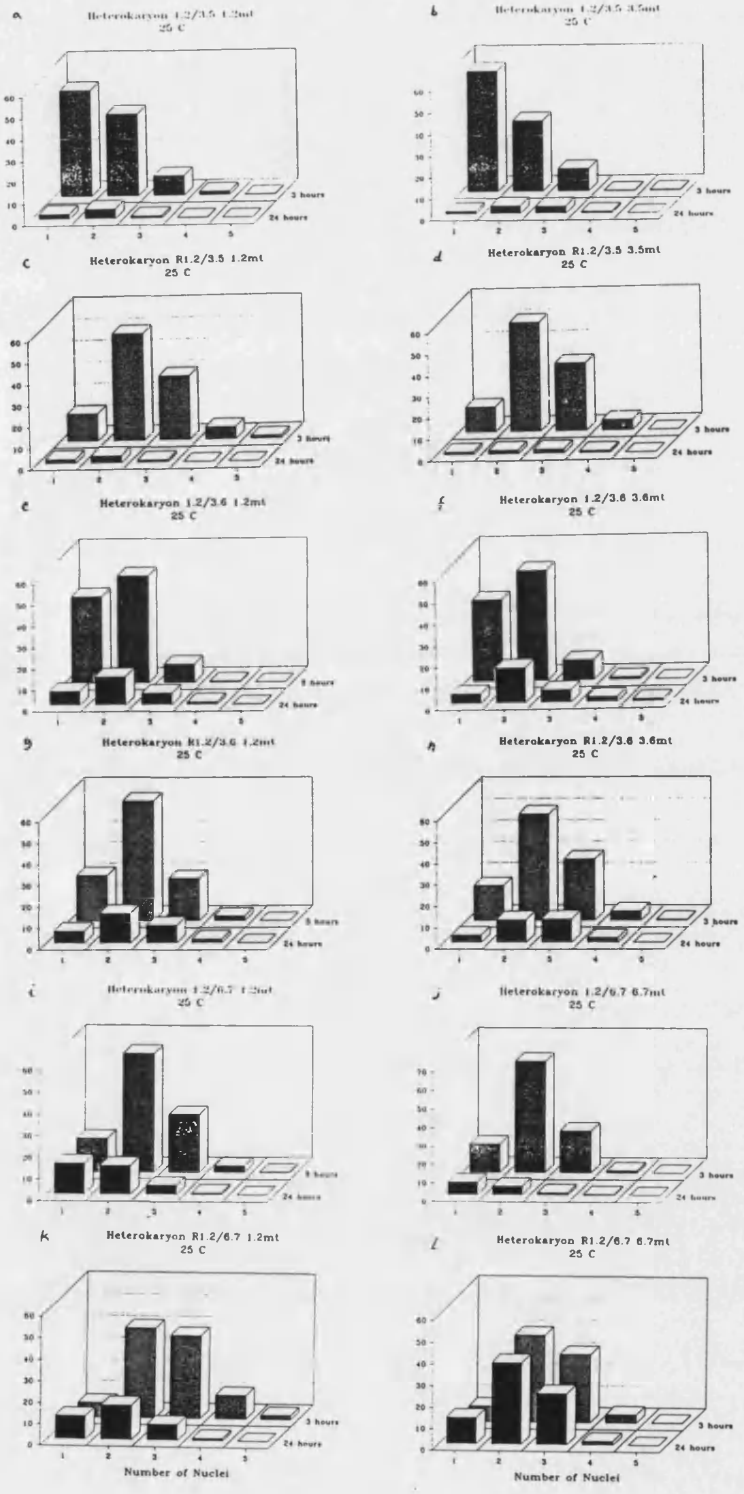


Figure 2.5. Distribution of numbers of nuclei in ungerminated conidia from 'original' and 'repeat' heterokaryotic isolates of *H.annosum* at 3 h and 24 h after plating out onto MEA at 20°C. Values shown are corrected for overall percentage germination (frequency at any given time x proportion of conidia not germinated). Sample sizes (number of conidia examined) for the 3 and 24 h sampling times were, respectively: (a) O1·2/3·5 1·2mt 20°C, (905, 853); (b) O1·2/3·5 3·5mt 20°C, (844, 710); (c) R1·2/3·5 1·2mt 20°C, (447, 310); (d) R1·2/3·5 3·5mt 20°C, (366, 446); (e) O1·2/3·6 1·2mt 20°C, (830, 733); (f) O1·2/3·6 3·6mt 20°C, (820, 791); (g) R1·2/3·6 1·2mt 20°C, (432, 327); (h) R1·2/3·6 3·6mt 20°C, (696, 473); (i) O1·2/6·7 1·2mt 20°C, (816, 800); (j) O1·2/6·7 6·7mt 20°C, (783, 746); (k) R1·2/6·7 1·2mt 20°C, (486, 368) and (l) R1·2/6·7 6·7mt 20°C, (498, 358).

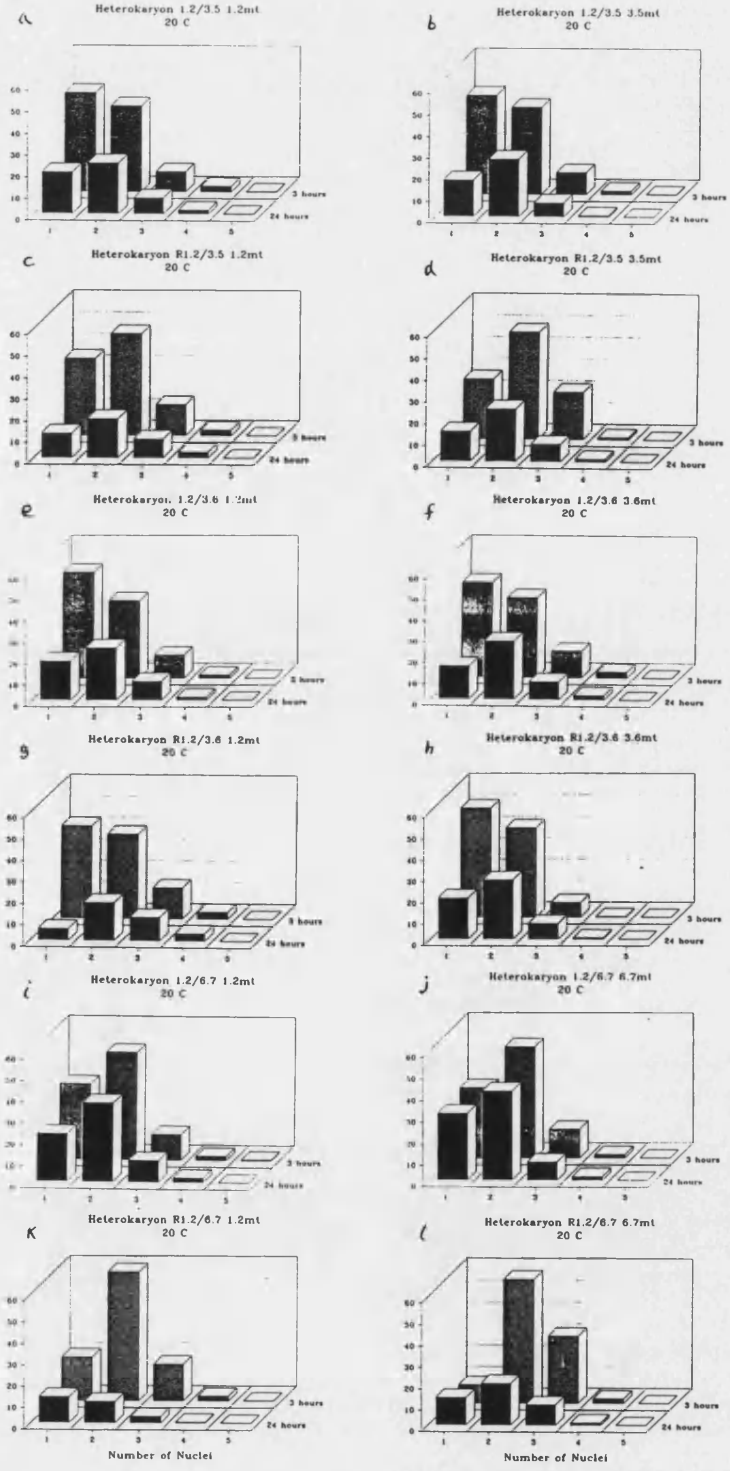


Figure 2.6. Distribution of numbers of nuclei in ungerminated conidia from 'original' and 'repeat' heterokaryotic isolates of *H.annosum* at 3 h and 24 h after plating out onto MEA at 15°C. Values shown are corrected for overall percentage germination (frequency at any given time x proportion of conidia not germinated). Sample sizes (number of conidia examined) for the 3 and 24 h sampling times were, respectively: (a) O1·2/3·5 1·2mt 15°C, (179, 116); (b) O1·2/3·5 3·5mt 15°C, (685, 284); (c) R1·2/3·5 1·2mt 15°C, (414, 261); (d) R1·2/3·5 3·5mt 15°C, (455, 702); (e) O1·2/3·6 1·2mt 15°C, (506, 235); (f) O1·2/3·6 3·6mt 15°C, (189, 235); (g) R1·2/3·6 1·2mt 15°C, (512, 498); (h) R1·2/3·6 3·6mt 15°C, (317, 335); (i) O1·2/6·7 1·2mt 15°C, (762, 223); (j) O1·2/6·7 6·7mt 15°C, (857, 206); (k) R1·2/6·7 1·2mt 15°C, (387, 316) and (l) R1·2/6·7 6·7mt 15°C, (339, 331).

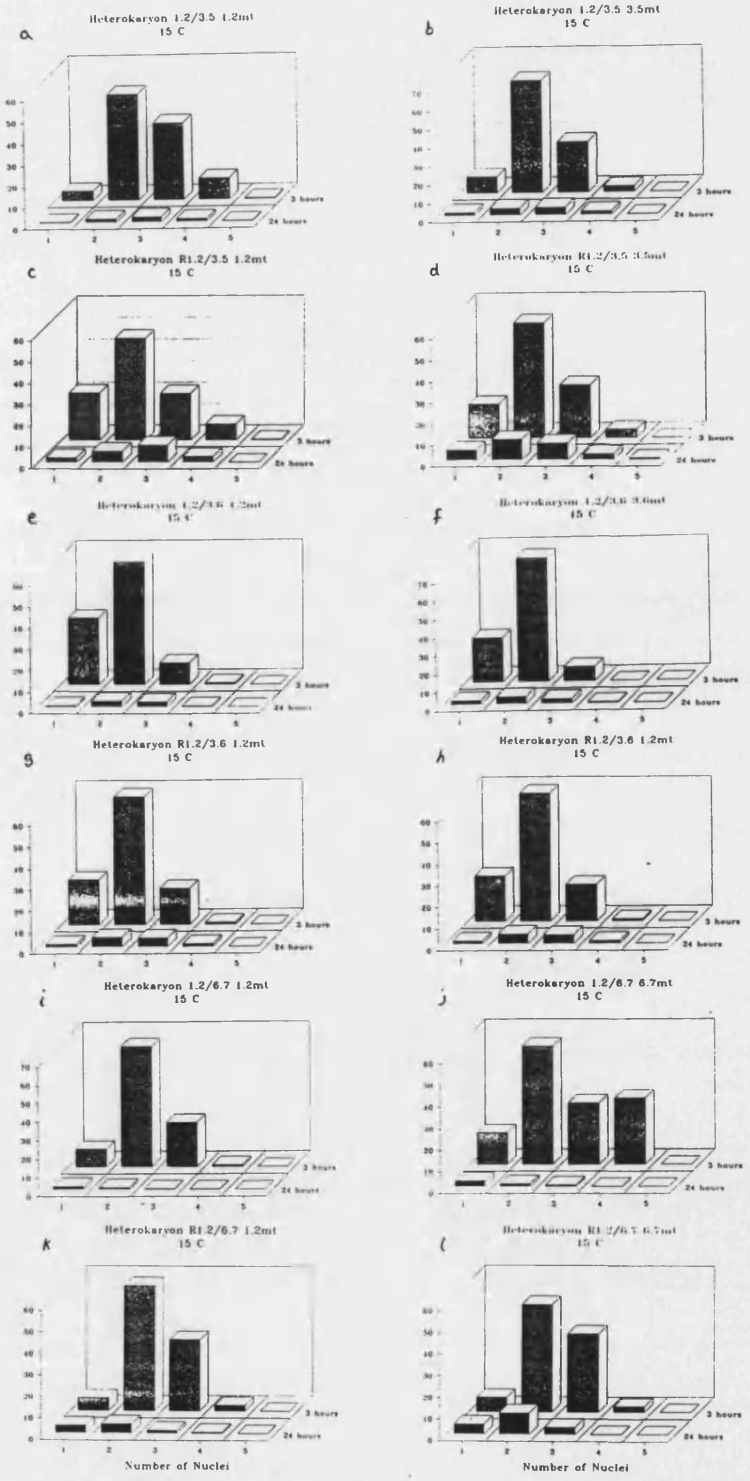


Figure 2.7. Distribution of numbers of nuclei in ungerminated conidia from 'repeat' heterokaryotic isolates of *H.annosum* at 3 h and 24 h after plating out onto MEA at 10°C. Values shown are corrected for overall percentage germination (frequency at any given time x proportion of conidia not germinated). Sample sizes (number of conidia examined) for the 3 and 24 h sampling times were, respectively: (a) O1·2/3·5 1·2mt 10°C, (625, 506); (b) O1·2/3·5 3·5mt 10°C, (401, 416); (c) R1·2/3·5 1·2mt 10°C, (417, 482); (d) R1·2/3·5 3·5mt 10°C, (399, 264); (e) O1·2/3·6 1·2mt 10°C, (354, 594); (f) O1·2/3·6 3·6mt 10°C, (599, 523); (g) R1·2/3·6 1·2mt 10°C, (422, 527); (h) R1·2/3·6 3·6mt 10°C, (465, 363); (i) O1·2/6·7 1·2mt 10°C, (473, 268); (j) O1·2/6·7 6·7mt 10°C, (593, 67); (k) R1·2/6·7 1·2mt 10°C, (359, 500) and (l) R1·2/6·7 6·7mt 10°C, (362, 444).

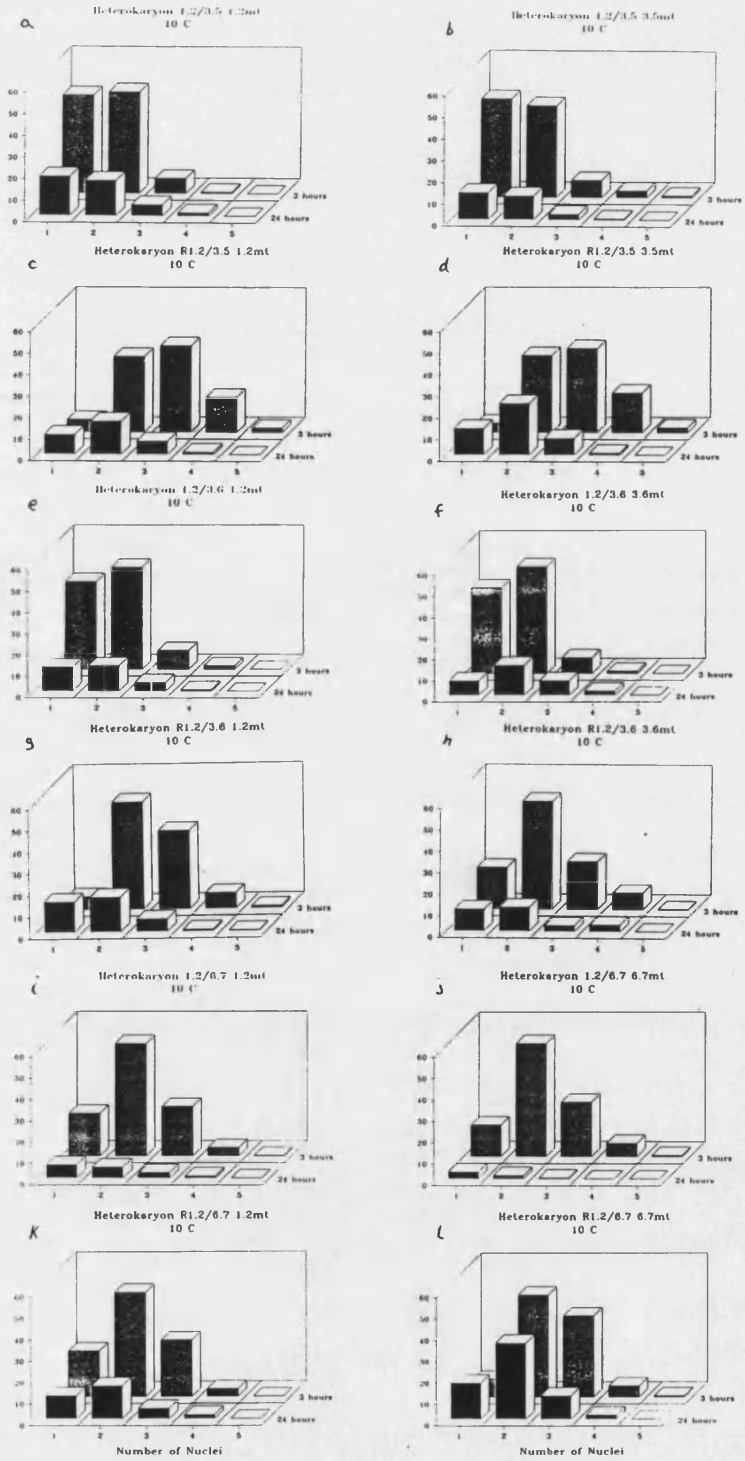


Table 2.3a. *Chi-squared, χ^2 (4 df), tests of the hypothesis that no differences in nuclear number distribution occur within two replicates for each incubation temperature / time / genotype combination.*

Original strains

Strain	25°C		15°C		10°C	
	3h	24h	3h	24h	3h	24h
1·2/3·5 1·2mt	2.241	4.812	-	2.611	6.932	7.04
	nsd	nsd		nsd	nsd	nsd
1·2/3·5 3·5mt	9.648	1.748	10.79	2.985	7.000	4.28
	*	nsd	*	nsd	nsd	nsd
1·2/3·6 1·2mt	1.884	4.701	0.879	3.427	0.661	2.18
	nsd	nsd	nsd	nsd	nsd	nsd
1·2/3·6 3·6mt	3.034	4.613	-	7.698	2.408	9.36
	nsd	nsd		nsd	nsd	nsd
1·2/6·7 1·2mt	16.62	6.510	1.556	1.186	4.722	0.53
	*	nsd	nsd	nsd	nsd	nsd
1·2/6·7 6·7mt	1.647	-	19.84	1.614	2.617	0.53
	nsd		*	nsd	nsd	nsd

P < *, 0.050; nsd, not significantly different.

Table 2.3b. *Chi-squared, χ^2 (4 df), tests of the hypothesis that no differences in nuclear number distribution occur within two replicates for each incubation temperature / time / genotype combination.*

Repeat strains

Strain	25°C		20°C		15°C		10°C	
	3h	24h	3h	24h	3h	24h	3h	24h
1·2/3·5 1·2mt	0.981	0.871	4.589	6.988	7.399	-	3.536	16.21
	nsd	nsd	nsd	nsd	nsd		nsd	**
1·2/3·5 3·5mt	3.649	0.312	-	1.364	4.325	30.79	2.013	5.277
	nsd	nsd		nsd	nsd	***	nsd	nsd
1·2/3·6 1·2mt	5.210	0.847	2.211	4.517	1.677	38.49	10.03	5.199
	nsd	nsd	nsd	nsd	nsd	***	*	nsd
1·2/3·6 3·6mt	5.153	4.627	5.727	1.190	10.02	6.457	20.89	12.19
	nsd	nsd	nsd	nsd	*	nsd	***	*
1·2/6·7 1·2mt	12.38	6.02	1.260	1.217	0.873	1.424	2.562	11.88
	*	nsd	nsd	nsd	nsd	nsd	nsd	*
1·2/6·7 6·7mt	3.365	1.026	0.483	1.500	2.934	0.461	5.149	3.481
	nsd	nsd	nsd	nsd	nsd	nsd	nsd	nsd

P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different.

Table 2.4a. *Chi-squared, χ^2 (4 df), tests of the hypothesis that nuclear number distributions in ungerminated conidia obtained from heterokaryons at different temperatures do not vary over time. Comparisons were made for combined replicate data against the initial frequencies at 3 h.*

Original strains

Strain	25°C 24h	20°C 24h	15°C 24h	10°C 24h
1·2/3·5 1·2mt	29.81 ***	-	22.97 ***	24.44 ***
1·2/3·5 3·5mt	141.4 ***	-	106.3 ***	5.401 nsd
1·2/3·6 1·2mt	68.21 ***	-	124.4 ***	9.043 nsd
1·2/3·6 3·6mt	112.1 ***	-	51.06 ***	99.71 ***
1·2/6·7 1·2mt	119.9 ***	-	240.8 ***	45.61 ***
1·2/6·7 6·7mt	89.62 ***	-	146.6 ***	83.98 ***

P < ***, 0.001; nsd, not significantly different.

Table 2.4b. *Chi-squared, χ^2 (4 df), tests of the hypothesis that nuclear number distributions in ungerminated conidia obtained from heterokaryons at different temperatures do not vary over time. Comparisons were made for combined replicate data against the initial frequencies at 3 h.*

Repeat strains

Strain	25°C 24 h	20°C 24 h	15°C 24 h	10°C 24 h
1·2/3·5 1·2mt	56.83 ***	15.73 **	60.41 ***	155.2 ***
1·2/3·5 3·5mt	67.16 ***	5.889 nsd	5.635 nsd	139.0 ***
1·2/3·6 1·2mt	20.22 ***	82.00 ***	131.8 ***	147.6 ***
1·2/3·6 3·6mt	23.36 ***	37.93 ***	97.24 ***	79.56 ***
1·2/6·7 1·2mt	99.56 ***	70.29 ***	119.9 ***	25.93 ***
1·2/6·7 6·7mt	18.28 **	65.40 ***	58.67 ***	75.51 ***

P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different.

Table 2.5. *Chi-squared, χ^2 (4 df), comparisons of nuclear number distributions in conidia from 'Original' and 'Repeat' heterokaryons at four temperatures, 10, 15, 20 and 25°C.*

Strain	25°C		20°C		15°C		10°C	
	3h	24h	3h	24h	3h	24h	3h	24h
1·2/3·5 1·2mt	177.8 ***	2.622 nsd	18.61 ***	18.02 **	33.30 ***	15.45 **	378.0 ***	28.77 ***
1·2/3·5 3·5mt	233.5 ***	13.56 **	51.44 ***	8.819 nsd	15.84 **	12.90 *	262.8 ***	28.18 ***
1·2/3·6 1·2mt	58.16 ***	14.44 **	9.976 *	68.24 ***	19.19 ***	7.703 nsd	191.8 ***	3.179 nsd
1·2/3·6 3·6mt	100.6 ***	62.83 ***	32.14 ***	11.19 *	8.417 nsd	24.86 ***	102.9 ***	69.19 ***
1·2/6·7 1·2mt	56.71 ***	24.63 ***	35.09 ***	29.67 ***	19.87 ***	16.57 **	11.79 *	11.21 *
1·2/6·7 6·7mt	43.70 ***	81.65 ***	127.2 ***	30.76 ***	21.58 ***	49.92 ***	21.24 ***	34.95 ***

P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different.

Table 2.6. *Chi-squared, χ^2 (12 or 8 df), tests comparing the nuclear number distribution patterns in conidia of heterokaryons at different temperatures (original 20°C data omitted).*

Strain	Original strains		Repeat strains	
	χ^2	P<(1)	χ^2	P<(2)
1·2/3·5 1·2mt	233.3	***	250.7	***
1·2/3·5 3·5mt	375.5	***	240.0	***
1·2/3·6 1·2mt	15.52	*	259.6	***
1·2/3·6 3·6mt	21.60	**	311.0	***
1·2/6·7 1·2mt	45.61	***	169.5	***
1·2/6·7 6·7mt	56.20	***	22.49	*

P < *, 0.050; **, 0.010; ***, 0.001. (1) 8df, (2) 12 df.

Quantitative comparisons of nuclear numbers in conidia from heterokaryons with identical nuclear genotypes, but different cytoplasms reveal statistically significant differences (Table 2.7a,b). Qualitatively, with the exception of strain R1.2/3.6 3.6mt at 25°C, both the patterns of nuclear allocation and subsequent germination of conidia were similar. A comparison of the overall levels of germination after 24 h, indicates that where a difference was detected, conidia with a 1.2 cytoplasmic genotype germinated most rapidly (9/13 comparisons). For the remaining 11 comparisons, no differences were detected.

The effect of temperature change upon overall germination rates was assessed for two heterokaryons and their derivative single-conidium derived homokaryotic progeny (Table 2.8). A temperature shift to 20°C produced an increase in the overall levels of germination in the majority of strains. Germination levels in homokaryons were generally high at all temperatures. Conidia from heterokaryotic mycelia germinated more rapidly as the incubation temperature increased. A marked decrease in germination rate was observed for all strains after they had been maintained at 25°C.

Genotype recovery

Genotype recovery patterns were examined in both the 'original' and 'repeat' heterokaryotic strains at four temperatures. Figure 2.8 illustrates a representative multi-well repli-dish containing single-conidium derived colonies taken from a heterokaryon of *H.annosum*. The presence of three distinct colony types can be detected along with wells where the germlings failed to develop. Data summarizing the genotypes recovered via conidia from heterokaryons are presented in Table 2.9. In 66 % of the heterokaryons examined (32/48), the recovery rates of homokaryotic genotypes were asymmetric, deviating significantly from a 1:1 ratio. A comparison of the observed recovery of heterokaryotic conidia with the expected, revealed that in 23 / 48 heterokaryons no significant differences were detected; for the remaining 25 strains, 13 produced fewer heterokaryons than expected. Of the 12 strains that produced more than expected most were from the repeat strains at low (10° and 15°C) temperatures.

When genotype recovery ratios from heterokaryons with different cytoplasmic partners were compared considerable differences were observed. This was the case for both the 'original' and 'repeat' experiments at all temperatures. 'Original' strains, O1.2/3.5 and O1.2/3.6, with

Figure 2.8. Repli-dishes containing single conidiospore isolates of *Heterobasidion annosum* showing parental homokaryotic, heterokaryotic and null (PGM) phenotypes. Plates were incubated for 4 wk at 20°C.

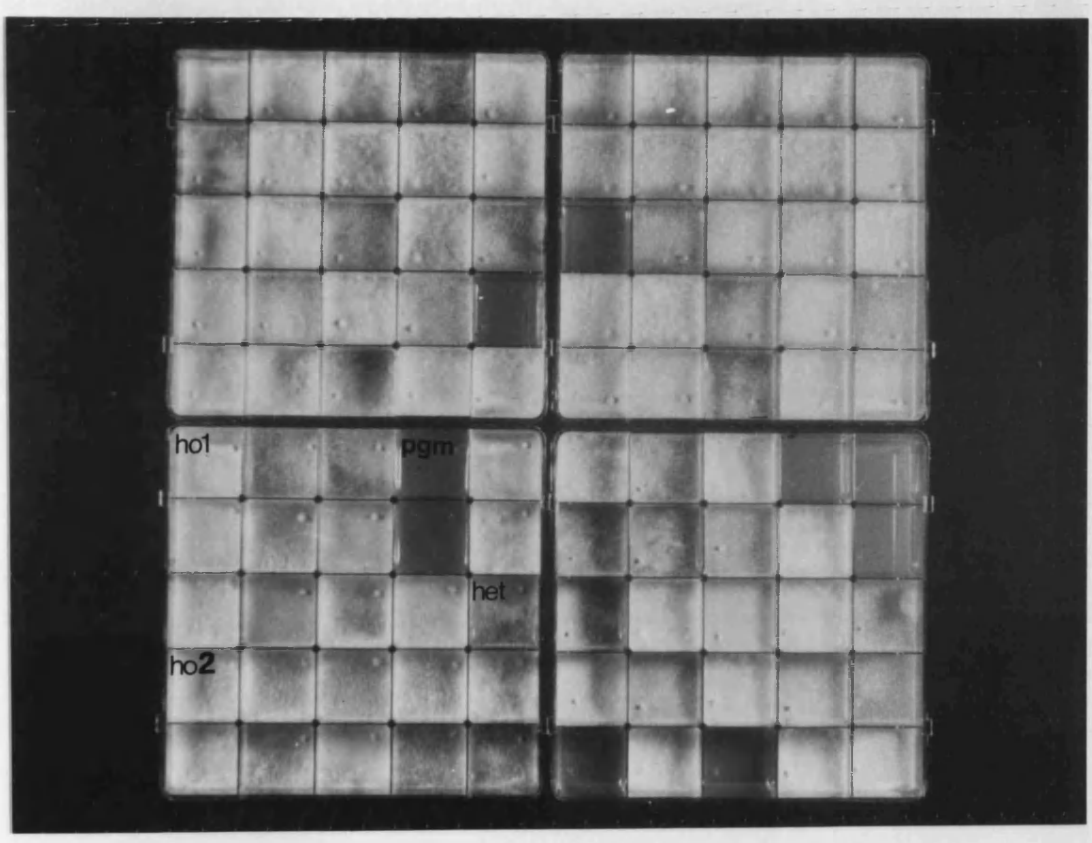


Table 2.7a. *Chi-squared, χ^2 (4 df), tests of the hypothesis that conidial nuclear number distributions do not differ between heterokaryons that contain identical nuclear genotypes but possess different mitochondrial genotypes. The data from two replicates are combined in each comparison. Separate comparisons were made for each temperature regime examined.*

Original strains

Strain	25°C		15°C		10°C	
	3h	24h	3h	24h	3h	24h
1·2/3·5	12.66	34.06	28.04	11.15	17.60	3.056
	*	***	***	*	**	nsd
1·2/3·6	1.416	23.93	7.511	15.69	1.543	48.48
	nsd	***	nsd	**	nsd	***
1·2/6·7	7.456	6.207	22.07	8.876	8.965	11.94
	nsd	nsd	***	nsd	nsd	*

P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different.

Table 2.7b. *Chi-squared, χ^2 (4 df), tests of the hypothesis that conidial nuclear number distributions do not differ between heterokaryons that contain identical nuclear genotypes but possess different mitochondrial genotypes. The data from two replicates are combined in each comparisons. Separate comparisons were made for each temperature regime examined.*

Repeat strains

Strain	25°C		20°C		15°C		10°C	
	3h	24h	3h	24h	3h	24h	3h	24h
1·2/3·5	4.47	93.06	12.86	9.713	14.611	25.559	1.473	2.642
	nsd	***	*	*	**	nsd	nsd	nsd
1·2/3·6	17.68	26.39	35.59	80.40	2.634	1.883	48.451	9.376
	**	***	***	***	nsd	nsd	***	nsd
1·2/6·7	17.95	28.15	45.09	29.87	2.652	23.87	31.70	10.10
	**	***	***	***	nsd	***	***	*

P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different.

different cytoplasmic partners, had large differences in nuclear proportions at low temperatures (10° and 15°C) whilst O1.2/6.7 heterokaryons, were more asymmetric at higher temperatures (20° and 25°C) - see Table 2.10a. Ratios within the 'repeat' heterokaryons were often more asymmetric than in the equivalent 'original' strains. Chi-squared values were generally higher than seen in the 'original' strain comparisons (Table 2.10b). The overall patterns observed were similar to those seen in the 'original' strains, but 1.2/6.7 heterokaryons demonstrated significant differences at all temperatures.

When strains from the 'original' and 'repeat' series were compared at each of the experimental temperatures, the least difference was observed at 20° and 25°C (Table 2.11). At lower temperatures genotype recovery rates were highly significantly different. The recovery of heterokaryotic genotypes was also differentially affected by the temperature treatments. At higher temperatures of 20° and 25°C, more heterokaryons were recovered from the 'repeat' strains than from the 'original' strains, whereas this pattern was reversed at 10°C .

Patterns of genotype recovery from a given heterokaryotic strain, as would be expected from the preceding descriptions, varied significantly at different temperatures (Table 2.12). The chi-squared levels for the 'original' strains were generally lower than those for the equivalent 'repeat' strains. For 'original' strains, O1.2/6.7 1.2mt, O1.2/6.7 6.7mt and O1.2/3.5 3.5mt, the variation between temperatures was low, or not significant.

Additional genotype recovery experiment

Genotype recovery data from heterokaryons generated by 'back-crossing' derived homokaryons at 20°C are presented in Table 2.13a. The genotype proportions were in general agreement with those observed in previous experiments. A comparison of equivalent heterokaryons with different cytoplasmic partners taken from the same side of a pairing (Table 2.13b), indicated that the cytoplasmic genotype had no influence over the dominance hierarchy.

Post-germination mortality

Post-germination mortality (PGM) levels in the strains of *H.annosum* examined were variable. A scatter plot of PGM levels in conidia from reciprocal heterokaryons (Figure 2.9), produced a linear relationship (Pearson's product moment correlation coefficient, r_p(22 df), equals 0.595; $P<0.005$), indicating that no clear relationship existed between mortality and the identity of the

Table 2.8. *Table showing effect of temperature shift on % germination. Measurements taken after 24 h incubation at the relevant temperature.*

Strain	10-10°	10-20°	15-15°	15-20°	25-25°	25-20°	20-20°
12N 12mt :12/35	86.4% (507)	93.9% (493)	83.3% (510)	92.1% (384)	21.8% (321)	32.3% (260)	96.7% (833)
12N 35mt :12/35	93.9% (580)	88.1% (548)	31.4% (522)	84.6% (427)	46.2% (569)	29.4% (531)	29.2% (377)
35N 35mt :12/35	81.0% (511)	94.8% (327)	89.4% (443)	99.2% (256)	21.9% (506)	20.9% (388)	12.4% (314)
35N 12mt :12/35	19.6% (484)	72.8% (313)	46.3% (244)	83.0% (312)	64.0% (247)	42.4% (238)	68.2% (311)
12/35 12mt	8.8% (715)	83.0% (452)	66.6% (98)	94.1% (220)	86.7% (271)	83.7% (368)	72.2% (425)
12/35 35mt	9.6% (743)	83.0% (417)	42.0% (326)	86.4% (353)	90.8% (305)	85.4% (371)	81.0% (388)

Numbers in parentheses indicate sample sizes.

Table 2.9. *Recovery of homokaryotic and heterokaryotic conidia from heterokaryons of Heterobasidion annosum maintained and generated at different temperatures.*

Original strains (25°C)

Strain	Conidia	*n*	%A	%B	χ^2_1	P<	%C	%D	χ^2_2	P<	PGM
1·2/3·5	1·2	25	26	31	11.6	***	35	-	-	-	44
1·2mt	3·5	55	57	69	-	-	65	-	-	-	-
	Het	16	17	-	-	-	-	24	3.19	nsd	-
1·2/3·5	1·2	20	19	26	18.1	***	30	-	-	-	47
3·5mt	3·5	56	54	74	-	-	70	-	-	-	-
	Het	28	27	-	-	-	-	19	3.69	nsd	-
1·2/3·6	1·2	25	19	23	35.4	***	29	-	-	-	33
1·2mt	3·6	85	64	77	-	-	71	-	-	-	-
	Het	22	17	-	-	-	-	22	2.41	nsd	-
1·2/3·6	1·2	20	14	18	53.7	***	24	-	-	-	31
3·6mt	3·6	94	68	82	-	-	76	-	-	-	-
	Het	25	18	-	-	-	-	18	0.00	nsd	-
1·2/6·7	1·2	37	22	62	3.31	nsd	55	-	-	-	20
1·2mt	6·7	23	14	38	-	-	45	-	-	-	-
	Het	107	64	-	-	-	-	58	2.59	nsd	-
1·2/6·7	1·2	30	22	42	1.71	nsd	46	-	-	-	16
6·7mt	6·7	41	31	58	-	-	54	-	-	-	-
	Het	63	47	-	-	-	-	51	0.85	nsd	-

Repeat strains (25°C)

Strain	Conidia	*n*	%A	%B	χ^2_1	P<	%C	%D	χ^2_2	P<	PGM
1·2/3·5	1·2	14	19	36	3.16	nsd	43	-	-	-	63
1·2mt	3·5	25	34	64	-	-	57	-	-	-	-
	Het	35	47	-	-	-	-	54	1.33	nsd	-
1·2/3·5	1·2	3	4	10	24.1	***	24	-	-	-	59
3·5mt	3·5	28	34	90	-	-	76	-	-	-	-
	Het	52	62	-	-	-	-	40	17.1	***	-
1·2/3·6	1·2	12	8	12	67.5	***	23	-	-	-	27
1·2mt	3·6	88	88	83	-	-	77	-	-	-	-
	Het	40	29	-	-	-	-	31	0.40	nsd	-
1·2/3·6	1·2	15	15	12	58.1	***	27	-	-	-	38
3·6mt	3·6	87	85	70	-	-	73	-	-	-	-
	Het	22	18	-	-	-	-	38	25.3	***	-
1·2/6·7	1·2	45	24	75	16.0	***	59	-	-	-	5
1·2mt	6·7	15	8	25	-	-	41	-	-	-	-
	Het	130	68	-	-	-	-	70	0.22	nsd	-
1·2/6·7	1·2	82	45	84	50.1	***	61	-	-	-	9
6·7mt	6·7	16	9	16	-	-	39	-	-	-	-
	Het	84	46	-	-	-	-	75	63.4	***	-

n, number of conidia isolated; %A, overall genotype percentage; %B, homokaryotic genotype percentage, χ^2_1, chi-squared (1 df) comparing the observed ratios of homokaryotic genotypes with a 1:1 ratio; %C, calculated mycelial nuclear percentage, %D, expected percentage of heterokaryons, χ^2_2, chi-squared (1 df) comparing the oberved and expected recovery of heterokaryons. P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different.

Table 2.9 continued. *Recovery of homokaryotic and heterokaryotic conidia from heterokaryons of Heterobasidion annosum maintained and generated at different temperatures.*

Original strains (20°C)

Strain	Conidia	*n*	%A	%B	χ^2_1	P≤	%C	%D	χ^2_2	P≤	PGM
1·2/3·5	1·2	52	46	50	0.01	nsd	50	-	-	-	43
1·2mt	3·5	51	45	50	-	-	50	-	-	-	-
	Het	12	9	-	-	-	-	23	9.74	***	-
1·2/3·5	1·2	23	27	32	9.39	***	35	-	-	-	56
3·5mt	3·5	49	56	68	-	-	65	-	-	-	-
	Het	15	17	-	-	-	-	21	0.67	nsd	-
1·2/3·6	1·2	42	35	45	1.06	nsd	46	-	-	-	40
1·2mt	3·6	52	43	55	-	-	54	-	-	-	-
	Het	26	22	-	-	-	-	16	2.50	nsd	-
1·2/3·6	1·2	22	26	35	2.87	*	38	-	-	-	58
3·6mt	3·6	41	49	65	-	-	62	-	-	-	-
	Het	21	25	-	-	-	-	19	1.68	nsd	-
1·2/6·7	1·2	112	54	65	15.7	***	60	-	-	-	0
1·2mt	6·7	60	29	35	-	-	40	-	-	-	-
	Het	35	17	-	-	-	-	27	10.7	**	-
1·2/6·7	1·2	28	39	46	0.41	nsd	48	-	-	-	68
6·7mt	6·7	33	46	54	-	-	52	-	-	-	-
	Het	11	15	-	-	-	-	45	25.9	***	-

Repeat strains (20°C)

Strain	Conidia	*n*	%A	%B	χ^2_1	P≤	%C	%D	χ^2_2	P≤	PGM
1·2/3·5	1·2	17	28	40	1.54	nsd	43	-	-	-	69
1·2mt	3·5	25	42	60	-	-	57	-	-	-	-
	Het	18	30	-	-	-	-	31	0.03	nsd	-
1·2/3·5	1·2	12	15	40	1.21	nsd	44	-	-	-	61
3·5mt	3·5	18	23	60	-	-	56	-	-	-	-
	Het	48	62	-	-	-	-	43	10.7	**	-
1·2/3·6	1·2	23	17	21	37.9	***	25	-	-	-	32
1·2mt	3·6	84	61	79	-	-	75	-	-	-	-
	Het	30	22	-	-	-	-	16	3.10	nsd	-
1·2/3·6	1·2	23	20	35	5.68	*	37	-	-	-	43
3·6mt	3·6	42	37	65	-	-	63	-	-	-	-
	Het	50	43	-	-	-	-	13	52.7	***	-
1·2/6·7	1·2	113	61	85	84.9	***	75	-	-	-	7
1·2mt	6·7	16	9	12	-	-	25	-	-	-	-
	Het	55	30	-	-	-	-	38	5.39	*	-
1·2/6·7	1·2	28	15	32	12.0	***	43	-	-	-	4
6·7mt	6·7	60	31	68	-	-	57	-	-	-	-
	Het	104	54	-	-	-	-	63	6.17	*	-

n, number of conidia isolated; %A, overall genotype percentage; %B, homokaryotic genotype percentage, χ^2_1, chi-squared (1 df) comparing the observed ratios of homokaryotic genotypes with a 1:1 ratio; %C, calculated mycelial nuclear percentage, %D, expected percentage of heterokaryons, χ^2_2, chi-squared (1 df) comparing the oberved and expected recovery of heterokaryons. P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different.

Table 2.9 continued. *Recovery of homokaryotic and heterokaryotic conidia from heterokaryons of Heterobasidion annosum maintained and generated at different temperatures.*

Original strains (15°C)

Strain	Conidia	*n*	%A	%B	χ^2_1	P<	%C	%D	χ^2_2	P<	PGM
1·2/3·5	1·2	6	8	14	24.6	***	30	-	-	-	62
1·2mt	3·5	36	51	86	-	-	70	-	-	-	-
	Het	29	41	-	-	-	-	51	2.95	nsd	-
1·2/3·5	1·2	6	12	14	24.6	***	28	-	-	-	75
3·5mt	3·5	36	72	86	-	-	72	-	-	-	-
	Het	8	16	-	-	-	-	43	17.5	***	-
1·2/3·6	1·2	30	33	45	0.55	nsd	47	-	-	-	48
1·2mt	3·6	36	40	55	-	-	53	-	-	-	-
	Het	24	27	-	-	-	-	36	3.64	nsd	-
1·2/3·6	1·2	60	42	52	0.18	nsd	51	-	-	-	28
3·6mt	3·6	56	39	48	-	-	49	-	-	-	-
	Het	28	19	-	-	-	-	39	26.6	***	-
1·2/6·7	1·2	37	22	52	0.13	nsd	51	-	-	-	9
1·2mt	6·7	34	20	48	-	-	49	-	-	-	-
	Het	101	58	-	-	-	-	52	3.14	nsd	-
1·2/6·7	1·2	22	19	35	5.33	*	43	-	-	-	22
6·7mt	6·7	40	34	65	-	-	67	-	-	-	-
	Het	54	47	-	-	-	-	47	0.00	nsd	-

Repeat strains (15°C)

Strain	Conidia	*n*	%A	%B	χ^2_1	P<	%C	%D	χ^2_2	P<	PGM
1·2/3·5	1·2	192	99	100	256	***	100	-	-	-	6
1·2mt	3·5	0	0	0	-	-	0	-	-	-	-
	Het	2	1	-	-	-	-	0	4.02	*	-
1·2/3·5	1·2	5	7	12	28.5	***	25	-	-	-	59
3·5mt	3·5	37	47	88	-	-	75	-	-	-	-
	Het	36	46	-	-	-	-	36	3.32	nsd	-
1·2/3·6	1·2	100	59	69	22.6	***	62	-	-	-	16
1·2mt	3·6	44	26	31	-	-	38	-	-	-	-
	Het	25	15	-	-	-	-	40	69.5	***	-
1·2/3·6	1·2	34	24	34	10.5	**	40	-	-	-	29
3·6mt	3·6	66	46	66	-	-	60	-	-	-	-
	Het	42	30	-	-	-	-	35	1.91	nsd	-
1·2/6·7	1·2	187	98	100	249	***	100	-	-	-	5
1·2mt	6·7	0	0	0	-	-	0	-	-	-	-
	Het	3	2	-	-	-	-	0	6.05	*	-
1·2/6·7	1·2	0	0	0	179	***	0	-	-	-	4
6·7mt	6·7	134	83	100	-	-	100	-	-	-	-
	Het	28	17	-	-	-	-	0	61.3	***	-

n, number of conidia isolated; %A, overall genotype percentage; %B, homokaryotic genotype percentage, χ^2_1, chi-squared (1 df) comparing the observed ratios of homokaryotic genotypes with a 1:1 ratio; %C, calculated mycelial nuclear percentage, %D, expected percentage of heterokaryons, χ^2_2, chi-squared (1 df) comparing the oberved and expected recovery of heterokaryons. P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different.

Table 2.9 continued. *Recovery f homokaryotic and heterokaryotic conidia from heterokaryons of Heterobasidin annosum maintained and generated at different temperatures*

Original trains (10°C)

Strain	Conidia	*n*	%A	%B	χ^2_1	P<	%C	%D	χ^2_2	P<	PGM
1·2/3·5	1·2	32	45	38	0.52	nsd	46	-	-	-	54
1·2mt	3·5	38	45	55	-	-	54	-	-	-	-
	Het	14	17	-	-	-	-	27	5.26	*	-
1·2/3·5	1·2	11	15	19	25.7	***	26	-	-	-	63
3·5mt	3·5	48	64	81	-	-	74	-	-	-	-
	Het	16	21	-	-	-	-	24	0.30	nsd	-
1·2/3·6	1·2	62	54	66	9.83	**	61	-	-	-	30
1·2mt	3·6	32	28	34	-	-	39	-	-	-	-
	Het	20	18	-	-	-	-	30	9.76	**	-
1·2/3·6	1·2	27	28	43	1.29	nsd	45	-	-	-	52
3·6mt	3·6	36	37	57	-	-	55	-	-	-	-
	Het	34	35	-	-	-	-	25	4.67	*	-
1·2/6·7	1·2	14	12	35	3.68	nsd	42	-	-	-	22
1·2mt	6·7	26	21	65	-	-	58	-	-	-	-
	Het	82	67	-	-	-	-	48	18.4	***	-
1·2/6·7	1·2	10	9	25	10.7	**	36	-	-	-	37
6·7mt	6·7	30	29	75	-	-	64	-	-	-	-
	Het	75	65	-	-	-	-	49	12.3	***	-

Repeat strains (10°C)

Strain	Conidia	*n*	%A	%B	χ^2_1	P≤	%C	%D	χ^2_2	P≤	PGM
1·2/3·5	1·2	192	100	100	256	***	100	-	-	-	4
1·2mt	3·5	0	0	0	-	-	0	-	-	-	-
	Het	0	0	-	-	-	-	0	0.00	nsd	-
1·2/3·5	1·2	0	0	0	245	***	0	-	-	-	5
3·5mt	3·5	184	97	100	-	-	100	-	-	-	-
	Het	6	3	-	-	-	-	0	12.2	***	-
1·2/3·6	1·2	182	99	100	242	***	100	-	-	-	7
1·2mt	3·6	0	0	0	-	-	0	-	-	-	-
	Het	2	1	-	-	-	-	0	4.02	*	-
1·2/3·6	1·2	23	25	41	1.80	nsd	46	-	-	-	45
3·6mt	3·6	33	36	59	-	-	54	-	-	-	-
	Het	36	39	-	-	-	-	54	8.18	**	-
1·2/6·7	1·2	40	22	53	0.21	nsd	52	-	-	-	16
1·2mt	6·7	36	21	47	-	-	48	-	-	-	-
	Het	102	57	-	-	-	-	51	2.84	nsd	-
1·2/6·7	1·2	0	0	0	213	***	0	-	-	-	4
6·7mt	6·7	160	88	100	-	-	100	-	-	-	-
	Het	22	12	-	-	-	-	0	46.8	***	-

n, number of conidia isolated; %A, overall genotype percentage; %B, homokaryotic genotype percentage, χ^2_1, chi-squared (1 df) comparing the observed ratios of homokaryotic genotypes with a 1:1 ratio; %C, calculated mycelial nuclear percentage, %D, expected percentage of heterokaryons, χ^2_2, chi-squared (1 df) comparing the oberved and expected recovery of heterokaryons. P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different.

Table 2.10a. *Chi-squared, χ^2, tests comparing the recovery of homokaryotic and heterokaryotic genotypes from different cytoplasmic backgrounds at different temperatures.*

Original Strains

Strain	Temp	Ho:Ho[1]	Ho:Het[1]	Ho:Ho:He[2]
1·2/3·5	10°C	10.56	0.564	11.08
		**	nsd	**
	15°C	0.000	8.531	8.531
		nsd	**	*
	20°C	5.949	1.982	7.856
		*	nsd	*
	25°C	0.462	3.060	3.523
		nsd	nsd	nsd
1·2/3·6	10°C	8.198	8.436	16.37
		**	**	***
	15°C	0.661	1.671	2.317
		nsd	nsd	nsd
	20°C	1.488	0.310	1.786
		nsd	nsd	nsd
	25°C	0.937	0.082	1.019
		nsd	nsd	nsd
1·2/6·7	10°C	0.556	0.503	1.059
		nsd	nsd	nsd
	15°C	7.642	0.276	7.964
		**	nsd	*
	20°C	6.932	0.103	7.100
		**	nsd	*
	25°C	13.73	0.002	13.73
		***	nsd	***

(1) 1 degree of freedom; (2) 2 degrees of freedom. P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different. Ho:Ho compares the recovery rates of component homokaryons, Ho:He compares the total number of homokaryons recovered with the number of heterokaryons, and Ho:Ho:He compares all three genotype classes.

Table 2.10b. *Chi-squared, χ^2, tests comparing the recovery of homokaryotic and heterokaryotic genotypes from different cytoplasmic backgrounds at different temperatures.*

Repeat Strains

Strain	Temp	Ho:Ho[1]	Ho:Het[1]	Ho:Ho:He[2]
1·2/3·5	10°C	376.0 ***	6.160 *	382.0 ***
	15°C	200.9 ***	94.25 ***	238.9 ***
	20°C	0.002 nsd	13.52 ***	13.52 ***
	25°C	6.458 *	3.732 nsd	10.13 **
1·2/3·6	10°C	124.51 ***	74.76 ***	175.6 ***
	15°C	29.95 ***	9.979 **	39.17 ***
	20°C	3.982 *	13.43 ***	17.21 ***
	25°C	0.319 nsd	4.292 *	4.612 nsd
1·2/6·7	10°C	120.51 ***	40.89 ***	159.6 ***
	15°C	251.1 ***	2.579 nsd	253.9 ***
	20°C	62.93 ***	29.64 ***	91.69 ***
	25°C	20.44 ***	0.098 nsd	20.54 ***

(1) 1 degree of freedom; (2) 2 degrees of freedom. P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different. Ho:Ho compares the recovery rates of component homokaryons, Ho:He compares the total number of homokaryons recovered with the number of heterokaryons, and Ho:Ho:He compares all three genotype classes.

Table 2.11. *Chi-squared, χ^2 (1 or 2 df), test comparisons of genotype recovery from 'Original' and 'Repeat' heterokaryons at four temperatures, 10, 15, 20 and 25°C.*

Strain	Temp	Ho:Ho[1]		Ho:Het[1]		Ho:Ho:He[2]	
		χ^2	P<	χ^2	P<	χ^2	P<
1·2/3·5 1·2mt	10°	121.9	***	33.71	***	146.4	***
1·2/3·5 3·5mt		35.93	***	23.34	***	55.89	***
1·2/3·6 1·2mt		70.08	***	27.88	***	94.52	***
1·2/3·6 3·6mt		0.039	nsd	0.337	nsd	0.376	nsd
1·2/6·7 1·2mt		0.529	nsd	3.990	*	4.528	nsd
1·2/6·7 6·7mt		104.8	***	16.38	***	118.8	***
1·2/3·5 1·2mt	15°	194.5	***	79.76	***	225.8	***
1·2/3·5 3·5mt		0.105	nsd	12.28	***	12.39	**
1·2/3·6 1·2mt		11.05	***	5.397	*	15.89	***
1·2/3·6 3·6mt		6.864	**	3.971	*	10.79	**
1·2/6·7 1·2mt		52.66	***	36.49	***	87.90	***
1·2/6·7 6·7mt		44.07	***	33.36	***	75.48	***
1·2/3·5 1·2mt	20°	1.198	nsd	10.63	**	11.72	**
1·2/3·5 3·5mt		0.610	nsd	34.19	***	34.70	***
1·2/3·6 1·2mt		12.29	***	0.002	nsd	12.30	**
1·2/3·6 3·6mt		0.003	nsd	7.222	**	7.226	*
1·2/6·7 1·2mt		0.175	nsd	5.793	*	5.968	nsd
1·2/6·7 6·7mt		12.35	***	6.794	**	20.25	***
1·2/3·5 1·2mt	25°	0.257	nsd	18.67	***	18.90	***
1·2/3·5 3·5mt		3.612	nsd	24.07	***	27.08	***
1·2/3·6 1·2mt		4.153	*	5.471	*	9.618	**
1·2/3·6 3·6mt		0.319	nsd	0.003	nsd	0.322	nsd
1·2/6·7 1·2mt		2.771	nsd	14.53	***	17.25	***
1·2/6·7 6·7mt		2.565	nsd	11.27	***	13.96	***

P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different. (1) 1 df; (2) 2 df. Ho:Ho compares the recovery rates of component homokaryons, Ho:He compares the total number of homokaryons recovered with the number of heterokaryons, and Ho:Ho:He compares all three genotype classes.

Table 2.12a. *Chi-squared, χ^2 (3 df), tests comparing genotype recovery from heterokaryons at different temperatures. Numbers of each homokaryotic genotype were examined.*

Strain	Original strains		Repeat strains	
	χ^2	P<	χ^2	P<
1·2/3·5 1·2mt	19.85	***	266.4	***
1·2/3·5 3·5mt	5.821	nsd	65.89	***
1·2/3·6 1·2mt	38.56	***	287.6	***
1·2/3·6 3·6mt	30.58	***	17.15	***
1·2/6·7 1·2mt	11.80	**	70.83	***
1·2/6·7 6·7mt	11.05	*	76.64	***

P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different

Table 2.12b. *Chi-squared, χ^2 (6 df), tests comparing the overall recovery of homokaryotic and heterokaryotic genotypes at different temperatures. Numbers of each homokaryotic and heterokaryotic genotype recovered were compared.*

Strain	Original strains		Repeat strains	
	χ^2	P<	χ^2	P<
1·2/3·5 1.2mt	45.33	***	372.6	***
1·2/3·5 3.5mt	9.607	nsd	194.9	***
1·2/3·6 1.2mt	42.30	***	340.8	***
1·2/3·6 3.6mt	43.16	***	39.06	***
1·2/6·7 1.2mt	18.71	**	113.3	***
1·2/6·7 6.7mt	20.57	**	211.4	***

P < **, 0.010; ***, 0.001; nsd, not significantly different

Table 2.13a. *Recovery of homokaryotic and heterokaryotic genotypes from heterokaryons generated by 'back-crossing' derived homokaryons of Heterobasidion annosum.*

Strain	Conidia	*n*	%A	%B	χ^2_1	P<	%C	%D	χ^2_2	P<	PGM
S1·2/6·7	1·2	27	36	51	0.00	nsd	50	-	-	-	28
3·5mt	6·7	26	35	49	-	-	50	-	-	-	-
	Het	21	28	-	-	-	-	29	0.03	nsd	-
S1·2/6·7	1·2	22	22	36	2.39	nsd	42	-	-	-	0
3·5mt	6·7	39	39	64	-	-	58	-	-	-	-
	Het	39	39	-	-	-	-	44	0.52	nsd	-
S1·2/6·7	1·2	36	43	57	0.64	nsd	55	-	-	-	15
6·7mt	6·7	27	33	43	-	-	45	-	-	-	-
	Het	20	24	-	-	-	-	29	0.64	nsd	-
S1·2/6·7	1·2	19	22	48	0.05	nsd	49	-	-	-	14
1·2mt	6·7	21	24	52	-	-	51	-	-	-	-
	Het	46	53	-	-	-	-	45	1.28	nsd	-

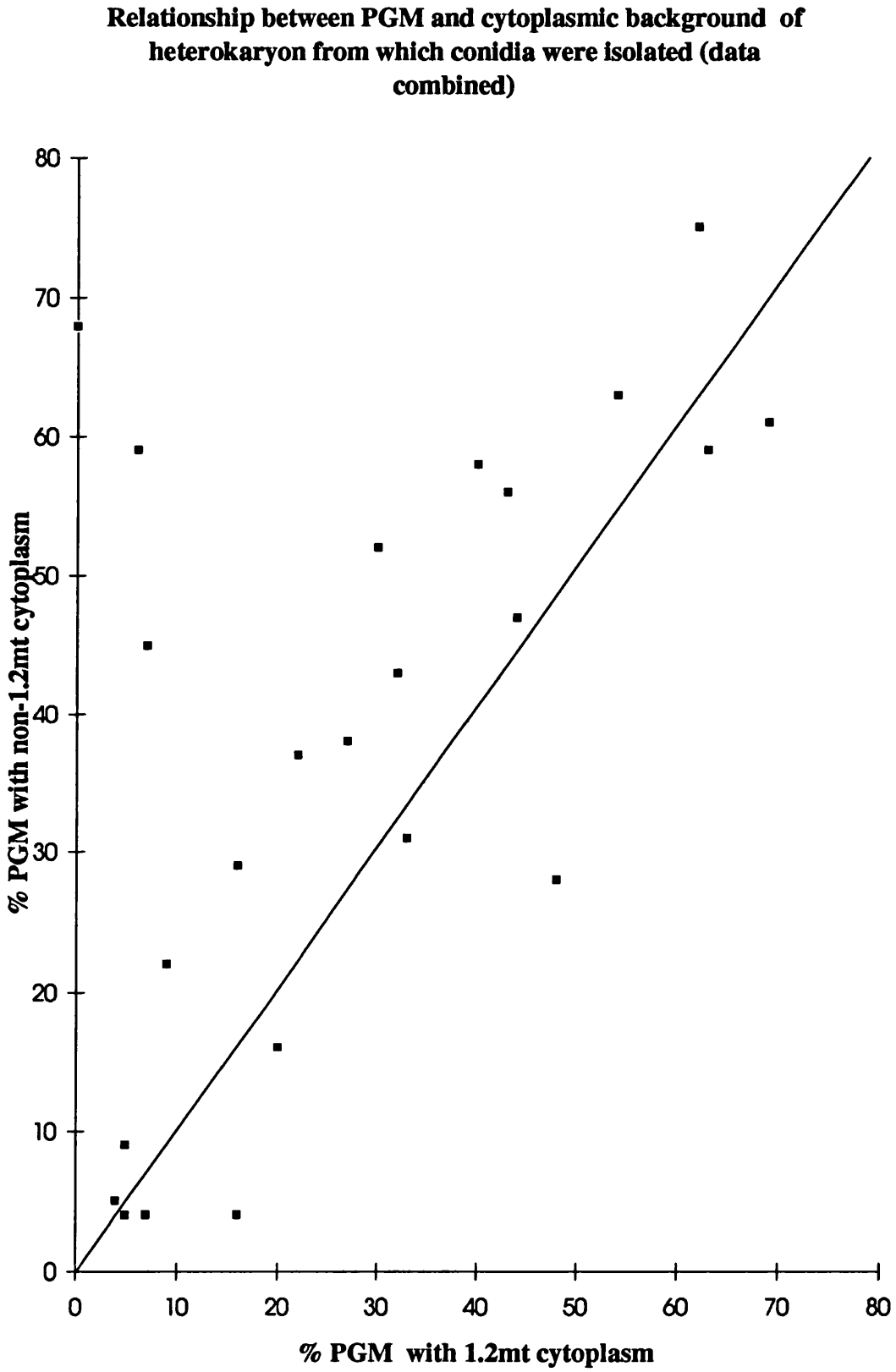
n, number of conidia isolated; %A, overall genotype percentage; %B, homokaryotic genotype percentage, χ^2_1, chi-squared (1 df) comparing the observed ratios of homokaryotic genotypes with a 1:1 ratio; %C, calculated mycelial nuclear percentage; %D, expected percentage of heterokaryons, χ^2_2, chi-squared (1 df) comparing the oberved and expected recovery of heterokaryons; PGM, percentage of germlings that fail to develop following transfer to MEA. P < nsd, not significantly different.

Table 2.13b. *Chi-squared, χ^2 (1 or 2 df), tests comparing genotype recovery from heterokaryons generated from 'back-crossed' homokaryons with identical nuclear genotypes and different cytoplasmic backgrounds. The table addresses the null hypothesis, cytoplasmic background has no effect on genotype recovery.*

Strain	Strain	Ho:Ho[1]	Ho:Het[1]	Ho:Ho:Het[2]
S1·2/6·7	S1·2/6·7	0.006	2.301	2.230
3·5mt	6·7mt	nsd	nsd	nsd
S1·2/6·7	S1·2/6·7	0.701	4.499	5.206
1·2mt	3·5mt	nsd	*	nsd

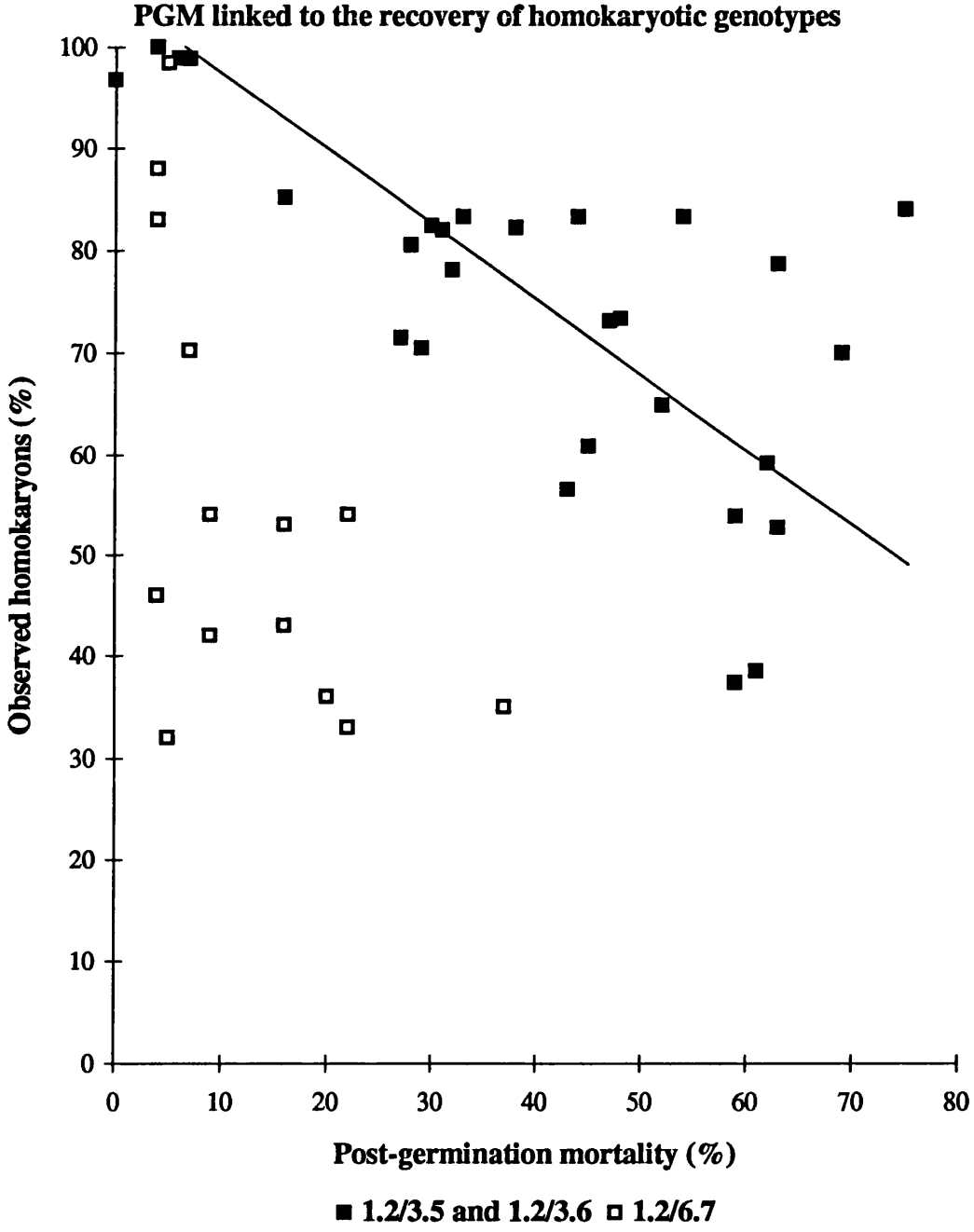
P < *, 0.050; nsd, not significantly different. (1) 1 df; (2) 2 df. Ho:Ho compares the recovery rates of component homokaryons, Ho:He compares the total number of homokaryons recovered with the number of heterokaryons, and Ho:Ho:He compares all three genotype classes.

Figure 2.9. Simple linear relationship between post-germination mortality and cytoplasmic background. PGM levels shown by germlings taken from conidia isolated from one side of an interaction plate are plotted against levels on the other side of the plate. Pearson's product moment correlation coefficient, r (22 df), equals 0.595 ($P<0.005$).



cytoplasmic genotype. When 'original' and 'repeat' data for the non sib-composed heterokaryons, 1.2/3.5 and 1.2/3.6 were combined, a strong relationship between the level of PGM and the observed recovery of homokaryotic genotypes was apparent (Figure 2.10). As the recovery of homokaryons increased, the levels of PGM mortality decreased (r_p(22 df), equals 0.607; P<0.001). In other words, a large proportion of heterokaryotic conidia fail to develop. Such a relationship was not found when 1.2/6.7 heterokaryons were examined. When mean post-germination mortality levels for all genotype / set-up time combinations were subjected to an analysis of variance, significant differences are found ($F_{(5,42\ df)} = 9.262$; $P < 0.01$). Duncan's multiple range test revealed that the 'original' and 'repeat' heterokaryons of 1.2/3.5 and 1.2/3.6 were not significantly different from each other ($\alpha = 0.05$); differences were detected between R1.2/6.7 and O1.2/6.7 strains. For all cases, mean PGM was lowest amongst germlings derived from the 'repeat' series strains.

Figure 2.10. (a) Relationship between PGM and the observed recovery rates for heterokaryons. 'Original' and 'repeat', 1.2/3.5 and 1.2/3.6 heterokaryon data for four temperatures were combined. Spearman's rank correlation coefficient, R_S (25 df), equals 0.607 ($P<0.001$). Also shown are the data for 1.2/6.7 heterokaryons.



DISCUSSION

The pattern of allocation of nuclei to conidia was in considerable agreement to those described in past studies. Only at the lowest temperatures was any deviation observed, with most heterokaryons producing higher numbers of binucleate conidia. This could reflect an adaptive response to the conditions (cf Huebschman, 1952), perhaps with the binucleate conidia possessing an advantage at low temperatures as a result of enhanced synthetic capacities relative to uninucleate conidia. Alternatively, developmental / metabolic conflicts may be reduced at low temperatures, a claim supported by the occurrence of similar effects on the expression of heterogenic incompatibility in *Podospora anserina* (Bernet, Bégueret & Labarère, 1973)

The recovery of genotypes via conidia from mycelia of laboratory-synthesized sympatric heterokaryons under a wide range of temperature regimes also followed similar patterns to those described in earlier sections. The importance of initial nuclear dominance, rather than cytoplasmic identity, as a factor affecting final genotype ratios was clarified. Furthermore, several new observations throw light onto the role played by 'invasiveness' as a primary determinant of final ratio.

Since most conidia from heterokaryotic mycelia germinated within a 24 h period at the high temperatures (20°C) normally used for genotype recovery evaluation, no significant bias would have been introduced into the data as a result of differential genotype selection during spore-picking. Although 25°C might have been a little better, the data concerning overall germinability lend additional confidence to any conclusions reached. Since the overall germinability (and presumably production of heterokaryotic conidia) was high in conidia obtained from heterokaryotic mycelia, post-germination failures arising from intergenomic conflicts still provide the most cogent explanation for the deficit in recovery of heterokaryotic mycelia and the observed levels of post-germination mortality.

Most cases where more (rather than fewer) heterokaryons were recovered than expected, were restricted to 'repeat' strains at low temperatures (10° and 15°C). Since the underlying nuclear ratios in these strains were often highly asymmetric (indeed several mycelia only produced one

homokaryotic genotype), 'hidden' genotypes would only be revealed in the few heterokaryons produced. Consequently on the basis of homokaryotic genotype ratios alone, the model used to predict expected heterokaryon recovery rates would indicate that no heterokaryotic conidia would be found on a mycelium that is composed of only one genotype. In general, the results still support the conclusion that fewer heterokaryons than expected were produced, and that intergenomic conflicts might be important determinants of population structure in *Heterobasidion.*

The relatively high levels of post-germination mortality amongst germlings from the unrelated non-sib composed heterokaryons 1.2 / 3.5 and 1.2 / 3.6 could be largely attributed to a loss of developmental competence amongst heterokaryotic conidia. The finding that post-germination mortality was lower in the 1.2 / 6.7 heterokaryotic combinations accords well with previous suggestions that genomic conflict is likely to be lower in strains with a common ancestry. Furthermore, the PGM data also support the proposition, arising from studies of allopatrically derived heterokaryons (see next section), that highly divergent nuclear ratios can reduce the incidence of developmental conflicts.

When dominance relationships amongst genotypes were compared under different experimental regimes informative patterns emerged. For both the 'original' and 'repeat' heterokaryons, rates of genotype recovery at 20° and 25°C strongly supported the existence of the same genotype dominance hierarchy described in an earlier section; ie 3.6 > 3.5 > 1.2 >= 6.7. When genotype recovery rates were compared at lower temperatures, the hierarchy still applied for most of the 'original' strains. A different pattern was evident in the 'repeat' strains, where the 'resident' genotype was dominant, possibly reflecting a combination of at least two factors: firstly the initial numerical dominance of the resident genotype, and secondly the slow rate of heterokaryotization at low temperatures.

The variation in genotype recovery ratio detected amongst the original strains at different temperatures was lower than that observed between the repeat strains, but in most cases the variation was statistically significant - making interpretation rather difficult. Nuclear ratios in the original heterokaryons appear to be confined to a narrower range than in the repeat pairings

but it would be unsafe to assume that this is really the case. The case for, or against nuclear selection is therefore somewhat equivocal.

When the proportion of invasive genotypes within 'resident' mycelia of 'repeat' heterokaryons at low temperatures was compared with the proportion of the 'resident' genotype in the reciprocal heterokaryon, a clear pattern became apparent. Nuclei from B1.2 appeared to invade mycelium occupied by B3.5 nuclei more rapidly than B3.5 nuclei invaded B1.2 domain, and B1.2 invaded B3.6 more rapidly than B3.6 invaded B1.2. Moreover, the rate of access of B1.2 nuclei into B3.6 mycelium was greater than the rate of access of B1.2 nuclei into B3.5 domain. Similarly, when the relative invasiveness of strains B1.2 and B6.7 were compared, B6.7 invaded B1.2 domain more rapidly than B1.2 invaded B6.7. This pattern is the reverse of the final genotype recovery dominance hierarchy, implying that there is an inverse relationship between final dominance, and invasiveness.

As noted in the introduction many studies have indicated a direct relationship between growth rate and nuclear ratio (Jinks, 1952; Sidhu, 1983a,b; Raper, 1985; Typas & Heale, 1976; Wang 1980). In this study a more complex situation occurred. The growth rates of the parental homokaryons were only reliable predictors of final dominance in 56% (27/48) of pairings. A stronger inverse relationship was detected between growth rate and dominance when the data for the growth rates of the component homokaryons recovered from a heterokaryon were used as predictors. Slow growth rates were associated with high dominance in 75% (32/48) of pairings.

Two contrasting modes of nuclear invasion are conceivable during heterokaryon formation; 'nuclear proliferation' or 'silent infiltration'. In the 'nuclear proliferation' strategy a genotype might out-replicate its competitor. However, if rapid nuclear invasion was linked to high rates of replication, signalling molecules produced by an invading genotype might act in a *trans* fashion, initiating nuclear division in the resident nuclear population as well. The result would be two synchronized replicating nuclear populations, ultimately leading to a stale-mate siuation, somewhat balanced in favour of the resident nuclei. Conversely, in the 'silent infiltration' strategy, an unexpressed invader, that avoided replication in non-self domain could achieved considerable dominance if the transition to the expressed state simultaneously reduced

the viabilty of the resident nuclear population (cf later discussions concerning allopatric heterokaryons of *Heterobasidion* and *Stereum*). A report on the genetics of nuclear behaviour in *Schizophyllum* gives further support to the infiltration scenario (Raper, 1985).

B-factor deletion mutants of *Schizophyllum commune* have greater competitive abilities than wild-type strains, which in turn are more competitive than strains in which B-factor expression is constitutive (Raper, 1985). The B-factor may have a role in signalling between nuclei since molecular genetic studies have revealed that it contains putative pheromone and pheromone binding regions (Specht in Casselton & Kües, 1994; Vaillancourt & Raper, 1995). B-factors may therefore have a controlling influence over the division rates of populations of nuclei. Raper (1985) also noted in her study, that the dominance hierarchy that she observed for B-factors was reversed when they operated in the context of a common-A mating.

If A-factor expression is epistatic to B-factor expression, B-factor expression might operate to restrain mitosis in fully compatible matings, but increase it in common-A heterokaryons and homokaryons. When A-factor recognition is dysfunctional the B-factor could be silenced leading to an increased rate of proliferation of nuclei. This would lead to a reduced competitive ability, as invading nuclei would simultaneously stimulate the over-replication of the resident genotype. The discovery that B-factors suppress nuclear division during heterokaryon formation, and that nuclear division rates within protoplasts from dikaryons were lower than those within protoplasts from monokaryons (Raper, 1985) lends further weight to these suggestions.

The dominant genotypes in the strains back-crossed to different cytoplasmic partners reflected the side of the interaction from which the heterokaryons were taken. A comparison of equivalent heterokaryons with different cytoplasmic partners taken from the same side of a pairing, indicates that the cytoplasmic genotype has no influence over the dominance hierarchy. These observations support the view that asymmetries observed in heterokaryons are to a large extent modified by the initial numerical predominance of a nuclear genotype within a mycelium.

Large differences at some temperatures, in the germinability of conidia from the mitotically derived homokaryotic strains of a heterokaryon (eg 1.2N 1.2mt: 1.2/3.5 and 3.5 1.2mt :

1.2/3.5, see Table 2.8), suggests that significant bias in favour of the recovery of the more germinable genotype might be expected (particularly if a 1:1 ratio in the original mycelium was envisaged). Although this is logical, the actual asymmetries recorded here, as well as those described previously, were mostly biased towards the recovery of the less germinable component homokaryon (ie 3.5 > 1.2). The inverse relationship between germinability and competitive ability might be linked to the growth rate correlations that have already been highlighted. Numerical asymmetries between genotypes within mycelia of sympatrically-derived heterokaryons might be even greater than previously reported.

SECTION III

IMBALANCED NUCLEAR RATIOS, POST-GERMINATION MORTALITY AND PHENOTYPE-GENOTYPE RELATIONSHIPS IN ALLOPATRICALLY-DERIVED HETEROKARYONS OF *HETEROBASIDION ANNOSUM* (FR.) BREF.

INTRODUCTION

This study extends the work previously carried out on the inter-relationships between nuclei within laboratory-synthesized sympatric heterokaryons of *H.annosum* by focusing upon the repercussions of matings between homokaryons derived from diverse geographical locations. Differences between laboratory-synthesized sympatric and allopatric heterokaryons and naturally-occurring heterokaryons are examined with particular regard to the nuclear ratios within mycelia, the allocation of nuclei to conidia and the patterns of conidial germination. The effects of differences in the relatedness of the associated genomes within heterokaryons upon these characteristics are also examined. The results are not only likely to reveal the extent of disharmony between distantly related genomes (cf those of sympatric origin) but also the potential consequences of geographical transposition in this economically important fungus.

The consequences of mating between allopatric populations are of considerable biological interest in that they reveal the potential for diverse and unstable relationships to develop between disparate nuclear and mitochondrial genomes inhabiting the same protoplasm. In sympatric populations, the inter-relationships between genomes are subject to unremitting selection pressure. This pressure may result in strong co-evolutionary feedbacks associated with nonself-recognition and the limitation of conflict, but it will not affect organisms that do not normally encounter one another in the field.

Long distance transfer of fungi by man might have significant repercussions on the genetic structure of resident populations. Geographical transposition is one of the most important mechanisms likely to cause what has been termed 'episodic selection' (Brasier, 1987; Brasier, 1995). Its impact is likely to be particularly rapid and dramatic in fungi, as a consequence of the opportunistic life styles and high rates of reproductive turnover possible in natural populations of these organisms. In fact, recent pandemics of Dutch elm disease caused by *Ophiostoma ulmi* (Buisman) Nannf. and *Ophiostoma novo-ulmi* Brasier are thought to provide a good example of the repercussions of episodic selection following geographical transposition. In this case, the repercussions have included the emergence of what might be termed 'super-pathogens'.

Root and but rot caused by *Heterobasidion annosum* is likely to be aggravated in commercial plantations as a result of current forestry practices. Tree density, species uniformity, location and post-harvest treatment of infected tree stumps are all likely to have had a major impact on the genetic structure of populations of this fungus.

MATERIALS AND METHODS

Strains, pairings and sub-culturing

The origins of all the strains examined in this study are presented in Table 3.1. Unless otherwise stated, all strains were grown and paired as described in the first section. After 4 wk growth, small mycelial fragments were removed from either side of the interactions zones to establish two presumptive heterokaryons with common nuclear partnerships, but different cytoplasmic backgrounds. After 4 d growth on 0.02% MEA, single hyphal tips were excised according to the method of Butler (1984). Stock-cultures of all strains were checked for clamp cells and were then maintained until required at 4°C on MEA bijou slopes under sterile mineral oil (Fisons: specific gravity 0.86 - 0.89 g ml^{-1})

Allopatric heterokaryons were synthesized by pairing three of the British homokaryons (B1.2, B3.5 and B3.6) with 18 of the Finish and Estonian P-group homokaryons in all possible combinations. After assessing the interactions between them twelve hyphal-tip-derived, clamp-connection bearing heterokaryons (FC1-FC12) were chosen for further study. These strains were specifically chosen because of their composition, so that comparisons could be made between strains with different degrees of nuclear and cytoplasmic relatedness, and for the ease with which the components of the heterokaryon could be distinguished when separated from their heterokaryotic partnerships.

Nuclear numbers in conidia and, when appropriate, genotype ratios, were examined in the twelve laboratory-synthesized allopatric heterokaryons, the naturally-occurring heterokaryons and 15 of the homokaryons.

Nuclear numbers in conidia

Conidial suspensions were prepared from heterokaryotic mycelia as described earlier. Nuclear numbers in a minimum of 200 ungerminated conidia obtained from 7-d-old heterokaryons were assessed for each of two replicates and at five sampling times (3,6,12,24 and 48 h) after initial transfer of conidia to sterile grid-filters overlaying MEA. Nuclei were visualized by staining air-dried spores with DAPI (1 μg ml^{-1}).

Table 3.1. *Designation of Heterobasidion annosum strains examined.*

Strain	Name	Site	Source	Date	ISG	Status
B 1.2	MR	Wiltshire,	*Pinus*	1991	P	Homo
B 3.5, B 3.6		UK	*Pinus*	1991	P	Homo
KK 87034. TI	KK	Kiljava,	*Pinus*	1987	P	Het
KK 87034. 1,3,5,6		Finland	*Pinus*	1987	P	Homo
KK 87035. TI			*Pinus*	1987	P	Het
KK 87035. 3,5,6,9			*Pinus*	1987	P	Homo
KK 91112. TI		Abruka,	*Picea*	1991	P	Het
KK 91112. 1-4,5*		Estonia	*Pinus*	1991	P	Homo
KK 91116. TI		Sorve,	*Juniperus*	1991	P	Het
KK 91116. 1-3,5,6*		Estonia	*Juniperus*	1991	P	Homo
B2 TI	MR	Wiltshire	*Pinus*	1994	P	Het
B2.1-8		UK	*Pinus*	1994	P	Homo
WH1 2.8		Gloucester	*Tsuga*	1992	P	Het
WH1 4.1		UK	*Tsuga*	1992	P	Het
WH1 9.5			*Tsuga*	1992	P	Het
WH2 5.2			*Tsuga*	1992	P	Het
WH2 5.3			*Tsuga*	1992	P	Het
WH2 11.4			*Tsuga*	1992	P	Het
Fr 86	JS	Frossarbo,	*Picea*	1984	S	Het
Fr 125		Sweden	*Picea*	1984	S	Het
Fr 162			*Picea*	1984	S	Het
Fr 185			*Picea*	1984	S	Het
Fr 186			*Picea*	1984	S	Het
Fr 188			*Picea*	1984	S	Het
Fr 212			*Picea*	1984	S	Het
TC 1	TC	USA			P	Het

The twelve laboratory-synthesized allopatric heterokaryons (FC1-FC12) were generated as follows; (FC1) B1·2 x KK91116·3 1·2mt; (FC2) B1·2 x KK91116·3 16·3mt; (FC3) B3·5 x KK91116·3 3·5mt; (FC4) B3.5 x KK91116·3 16·3mt; (FC5) B3·6 x KK91116·3 3·6mt; (FC6) B3·6 x KK91116·3 16·3mt; (FC7) B1·2 x KK87034·1 1·2mt; (FC8) B3·5 x KK87034·1 3·5mt; (FC9) B3·6 x KK87034·1 3·6mt; (FC10) B3·6 x KK87034·3 3·6mt; (FC11) B3·6 x KK87034·5 3·6mt and (FC12) B3·6 x KK91112·1 3·6mt. (mt) refers to the side of the interaction from which the heterokaryon was recovered.

ISG = intersterility group classification. Collectors were; (MR) Mark Ramsdale, (JS) Jan Stenlid, (KK) Kari Korhonen and (TC) Tom Chase. * refers to homokaryons with a senescent morphology.

Percentage germination was assessed by counting the numbers of germinated and ungerminated conidia in separate random fields of view until a minimum of 500 spores had been examined.

Genotype recovery from heterokaryons

Spore suspensions were spread onto MEA plates at various dilutions. After 24 h incubation, *c.* 200 individual well-separated germlings were transferred to Repli plate wells (Bibby Sterilin Ltd) containing 3 ml MEA and incubated at 20°C for 4 wk. The number of germlings that failed to develop following transfer (post-germination-mortality, PGM) was recorded along with the numbers of colonies that could be identified as one or other of the original homokaryons (on the basis of the distinctive morphological characteristics for which they were chosen) or as a heterokaryon (on the basis of morphology and clamp-connection production). Pairings were set up between a sub-set of the conidium-derived strains and their progenitor homokaryons in order to assess the stability of their somato-sexual recognition responses.

Using the equations presented earlier, the ratios of nuclear genotypes within heterokaryotic mycelia and the expected number of heterokaryons were calculated.

Statistical analyses

Data for nuclear number distributions in ungerminated conidia were analyzed using χ^2 tests corrected for discontinuity when appropriate (Milton & Tsokos, 1983). Consistency of results was generally confirmed, so the replicate data were combined during later analysis.

The distribution of nuclei in ungerminated conidia within a strain for the different sampling times was compared to the distribution observed after 3 h. The observed ratios of nuclear genotypes within a mycelium were compared to that of a 1:1 ratio. The recovery of heterokaryons was compared with that expected from the model presented earlier.

PGM for each strain was compared to that shown by conidia recovered directly from the progenitor homokaryons. The relationships between PGM and recovery rates of specific genotypes were assessed using linear regression models and Spearman's rank correlation coefficient.

RESULTS

Strain characteristics and interaction outcomes

Regardless of geographical origin, most non-sib pairings amongst members of the same intersterility group were fully compatible, as indicated by the formation of clamp-connections. Those that failed to mate involved pairings between senescent strains (characterized by the production of appressed sienna mycelia, monilioid hyphae and the production of aromatic planar crystalline aggregates). Pairings between senescent strains and normal strains were mostly incompatible, but several instances of 'recovery' from senescence were observed when senescent strains were paired against allopatric homokaryons. Such recovery was never observed in sympatric pairings.

Heterokaryon formation between allopatric strains was accompanied by phenotypic changes in some cases, but often the clamp connection-bearing, hyphal-tip-derived heterokaryons resembled one parent more than the other. Some of the allopatric heterokaryons produced pseudosclerotial plates. Patterns of heterokaryon emergence varied considerably between combinations, but were found to be highly reproducible. The homokaryotic strains chosen for further study were morphologically distinct and were readily identifiable after recovery from heterokaryons; even when they were associated with an unfamiliar cytoplasmic background. This contrasted with the situation in many of the other artificial combinations examined and with 14 out of the 19 natural heterokaryons. In these, the recovered homokaryotic genotypes often exhibited little variation and could not therefore be reliably separated on the basis of morphological characteristics alone (cf Dickhardt, 1985).

Nuclear numbers in conidia

All allopatrically derived heterokaryons (except FC7) produced conidia that were initially mostly uninucleate (Figure 3.1). No significant differences in nuclear number distributions were detected amongst replicate samples from any of the strains. Germination percentages for conidia were high (mean 79 ± 5% after 48 h incubation). The distribution of the numbers of nuclei in ungerminated conidia at various times after inoculation changed characteristically. Uninucleate conidia germinated more rapidly than multinucleate conidia, resulting in a preponderance of binucleate conidia after 48 h. The relative rates at which uni-, bi- and

Figure 3.1. Distribution of numbers of nuclei in ungerminated conidia from allopatrically derived heterokaryotic isolates of *H.annosum* at different times after plating out onto MEA at 20°C. Values shown are corrected for overall percentage germination (frequency at any given time x proportion of conidia not germinated). Sample sizes (number of conidia examined) for the 3,6,12,24 and 48 h sampling times were, respectively: (a) FC1 (454, 356, 314, 447, 273); (b) FC2 (372, 348); (c) FC3 (370, 489, 338, 574, 244); (d) FC4 (327, 410, 465, 455, 211); (e) FC5 (516, 446, 408, 487, 282); (f) FC6 (336, 435, 428, 397, 377). Continued overleaf.

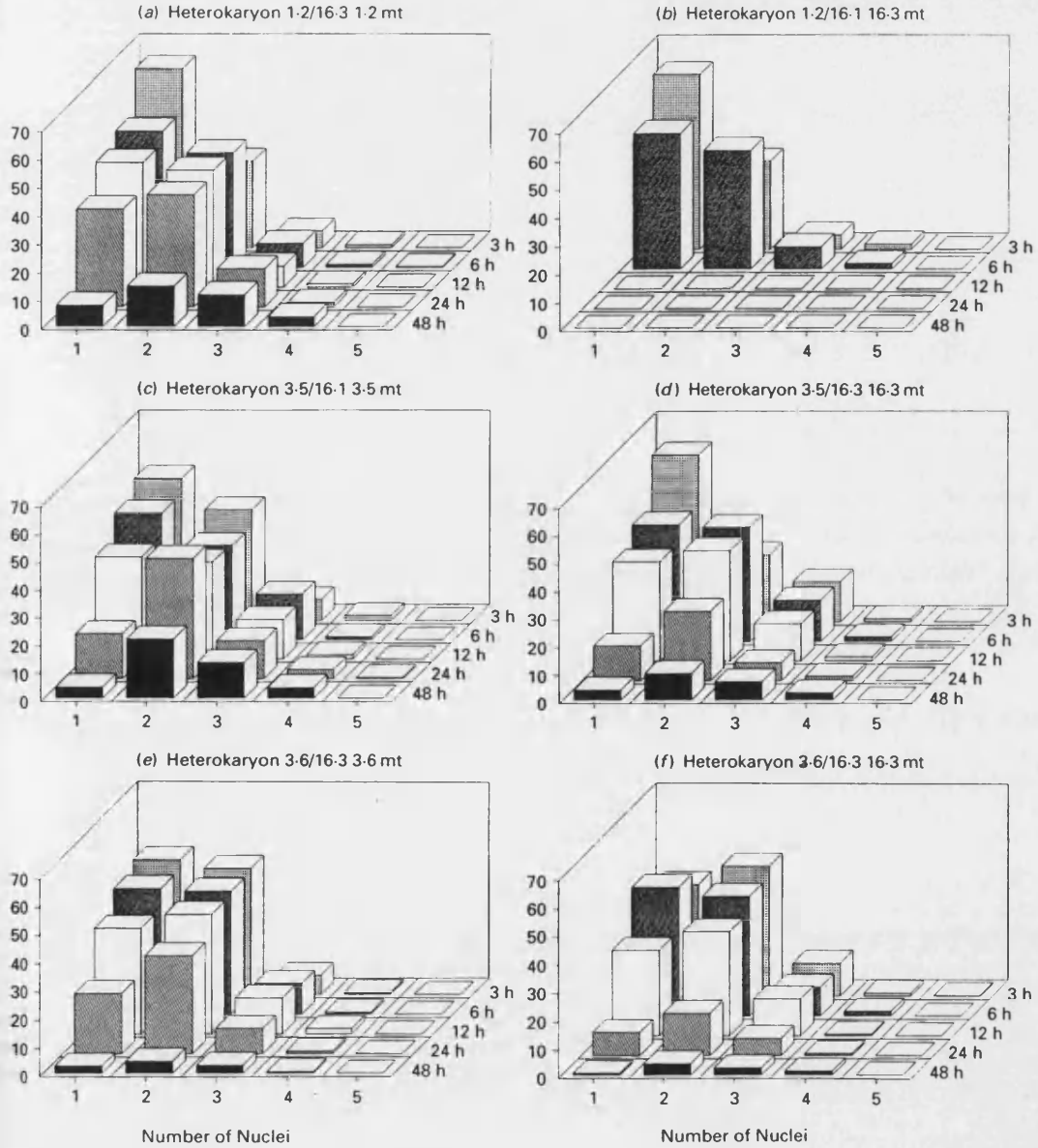
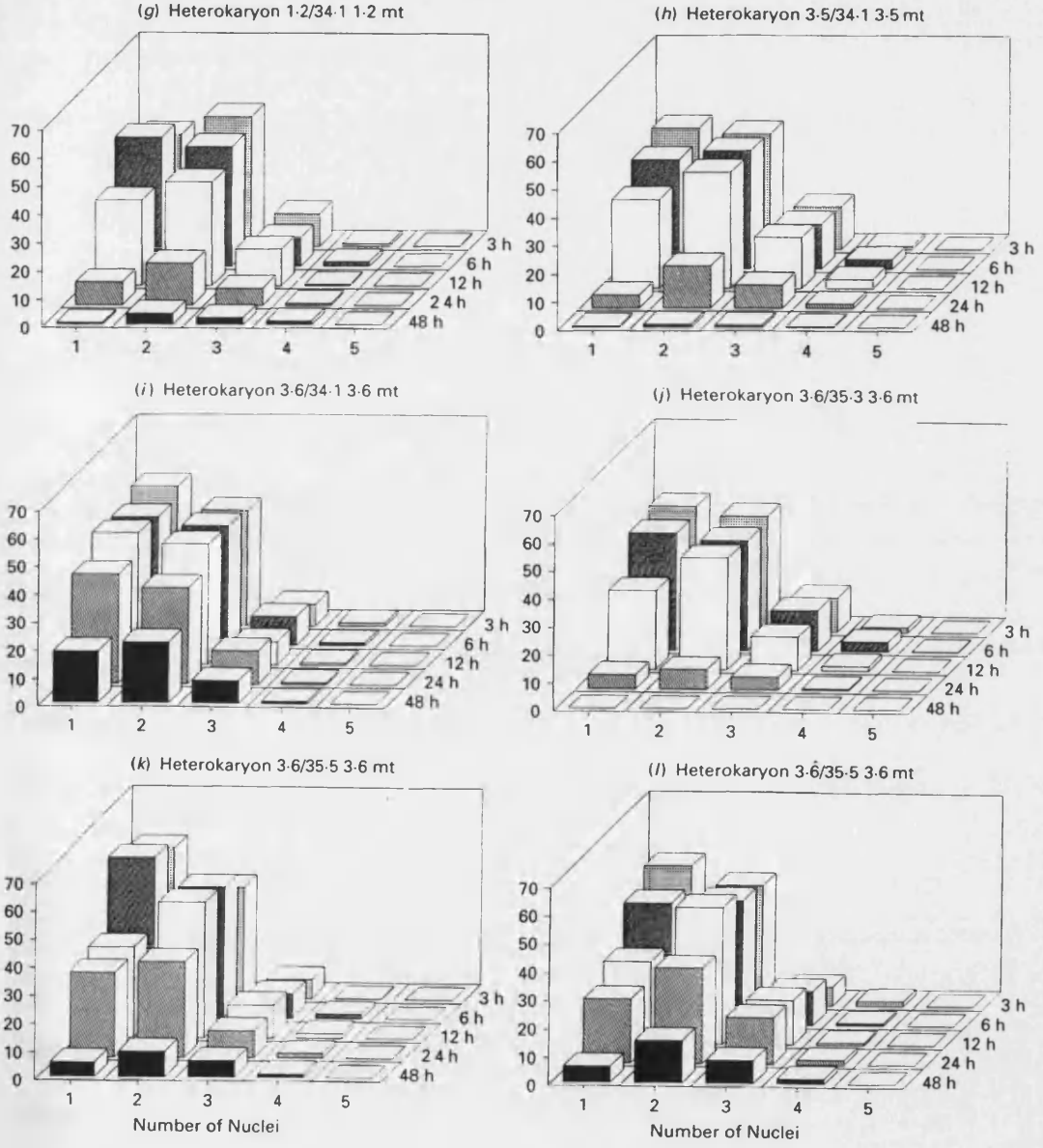


Figure 3.1 continued. Distribution of numbers of nuclei in ungerminated conidia from allopatrically derived heterokaryotic isolates of *H.annosum* at different times after plating out onto MEA at 20°C. Values shown are corrected for overall percentage germination (frequency at any given time x proportion of conidia not germinated). Sample sizes (number of conidia examined) for the 3,6,12,24 and 48 h sampling times were, respectively: (g) FC7 (380, 420, 1017, 567, 341); (h) FC8 (500, 455, 398, 279, 368); (i) FC9 (424, 441, 392, 402, 343); (j) FC10 (381, 522, 381, 426); (k) FC11 (468, 356, 382, 376, 315); FC12 (476, 661, 447, 314, 252).



multinucleate conidia germinated varied amongst the strains, but all the strains showed qualitatively similar changes. Chi-squared, χ^2 (4 df), comparisons indicated that the changes were highly significant (Table 3.2). Nuclear division within conidia following their delimitation and prior to germ-tube formation has not yet been observed. Consequently this could not account for the patterns of change described.

When the allocation of nuclei to conidia and their subsequent patterns of germination were compared, the greatest similarity occurred between heterokaryons with the same nuclear partners but different cytoplasms - 66 % of category 2 comparisons showed no significant differences, see Table 3.3). When heterokaryons had one partner in common and the others were sib-related, some similarity was observed, but the differences were greater when the cytoplasmic backgrounds differed - categories 3a and 3b. Greater differences were detected as both the nuclear and cytoplasmic genotypes of the heterokaryons became successively more disparate eg categories 4a - 5a (drop from 46% nsd to 23% nsd). When completely unrelated heterokaryons were compared, the overall level of similarity surprisingly rose to 41% and 46% - categories 5b and 6 respectively.

Nuclear numbers in conidia from natural heterokaryons were measured after 3 h and 24 h (Figure 3.2). Many (13 out of 15) natural heterokaryons produced conidia that were predominantly binucleate at 3 h. In most cases, statistically significant changes in distribution were observed over time (Table 3.4). In contrast with the allopatric heterokaryons, binucleate conidia germinated equally or more readily than uninucleate conidia.

Genotype recovery

The recovery rates for genotypes from allopatric heterokaryons are shown in Table 3.5. Homokaryotic genotypes were always more frequently recovered from the mycelium that they had invaded than from their resident mycelium, suggesting that they were also numerically dominant within the non-resident mycelium itself. The observed asymmetries were very large in some heterokaryons eg 9:1 for FC10, and they were generally greater than those observed in the laboratory-synthesized sympatric heterokaryons described previously. When the mean ratio asymmetries of laboratory-synthesized sympatric and allopatric heterokaryons were compared,

Table 3.2. *Chi-squared, χ^2 (4 df), tests of the hypothesis that nuclear number distributions in ungerminated conidia obtained from allopatric heterokaryons do not vary over time.*

Strain	6h	12h	24h	48h
FC1	15.93 **	18.36 **	55.20 ***	166.0 ***
FC2	14.37 **	-	-	-
FC3	16.82 **	15.31 **	99.32 ***	147.4 ***
FC4	27.15 ***	38.33 ***	95.09 ***	110.4 ***
FC5	5.487 nsd	17.59 **	31.15 ***	101.2 ***
FC6	0.833 nsd	5.455 nsd	14.35 **	117.5 ***
FC7	3.635 nsd	14.89 **	22.32 ***	138.5 ***
FC8	6.705 nsd	13.10 *	68.95 ***	114.6 ***
FC9	2.469 nsd	0.896 nsd	8.288 nsd	20.52 ***
FC10	3.998 nsd	8.358 nsd	35.80 ***	-
FC11	5.130 nsd	36.88 ***	24.56 ***	115.5 ***
FC12	12.01 *	44.34 ***	52.47 ***	99.91 ***

Comparisons were made for combined replicate data against the initial frequencies at 3 h. P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different

Table 3.3. *Distribution of numbers of nuclei within ungerminated conidia at 3, 6, 12, 24 and 48 h compared using the Chi-squared test, χ^2 (4df) in a pairwise fashion between FC1-FC12 strains with different degrees of relatedness.*

#	P<	3 h	6 h	12 h	24 h	48 h	Sum
1	nsd	12	12	12	12	12	(60)
	*	0	0	0	0	0	(0)
	**	0	0	0	0	0	(0)
	***	0	0	0	0	0	(0)
		(12)	(12)	(12)	(12)	(12)	(60)
2	nsd	1	3	3	1	1	(9)
	*	0	0	0	1	0	(1)
	**	1	0	0	0	0	(1)
	***	1	0	0	1	1	(3)
		(3)	(3)	(3)	(3)	(2)	(14)
3a	nsd	1	2	2	1	1	(7)
	*	0	0	0	0	1	(1)
	**	0	0	0	0	0	(0)
	***	1	0	0	1	0	(2)
		(2)	(2)	(2)	(2)	(2)	(10)
3b	nsd	1	1	2	1	2	(7)
	*	1	1	0	0	0	(2)
	**	0	1	0	0	0	(1)
	***	1	0	1	2	1	(5)
		(3)	(3)	(3)	(3)	(3)	(15)
4a	nsd	8	10	6	3	3	(30)
	*	1	0	2	1	1	(5)
	**	1	3	2	3	3	(12)
	***	5	2	2	5	5	(19)
		(15)	(15)	(12)	(12)	(12)	(66)
4b	nsd	3	6	1	3	2	(15)
	*	2	1	3	2	0	(8)
	**	0	1	2	1	1	(5)
	***	3	0	0	0	2	(5)
		(8)	(8)	(6)	(6)	(5)	(33)
4c	nsd	2	2	2	1	1	(8)
	*	1	2	1	0	0	(4)
	**	0	0	0	0	2	(2)
	***	1	0	1	3	1	(6)
		(4)	(4)	(4)	(4)	(4)	(20)
5a	nsd	5	1	3	0	0	(9)
	*	0	4	3	0	2	(10)
	**	0	3	1	1	1	(7)
	***	3	1	0	7	3	(14)
		(8)	(8)	(8)	(8)	(6)	(38)
5b	nsd	1	4	5	1	2	(13)
	*	0	2	1	1	0	(4)
	**	0	0	0	1	0	(1)
	***	5	0	0	3	3	(11)
		(6)	(6)	(6)	(6)	(5)	(29)
6	nsd	1	12	6	6	4	(29)
	*	2	1	2	1	1	(7)
	**	6	2	1	1	0	(10)
	***	7	1	2	3	4	(17)
		(16)	(16)	(11)	(11)	(9)	(63)

See overleaf for table legend.

Table 3.3 legend. P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different. Categories of relatedness between two heterokaryons were defined as follows; (1) identical nuclear partners, identical cytoplasms, (2) identical nuclear partners, cytoplasms differ, (3a) one partner in common, other partners sib-related, cytoplasms identical, (3b) one partner in common, other partners sib-related, cytoplasms differ, (4a) One partner in common, other partners non-sibs, identical cytoplasms, (4b) One partner in common, other partners are non-sibs, identical cytoplasms but from different sibs, (4c) one partner in common, other partners are non-sibs, different cytoplasms, (5a) all partners differ, one set are sib-related, identical cytoplasms from different sibs, (5b) All partners differ, one set are sibs, cytoplasms differ, (6) all partners differ, no sibs involved, cytoplasms differ.

Figure 3.2. Distribution of numbers of nuclei in ungerminated conidia from naturally-occurring heterokaryotic isolates of *H.annosum* at 3 and 24 h after plating out onto MEA at 20°C. Values shown are corrected for overall percentage germination (frequency at any given time x proportion of conidia not germinated). Sample sizes (number of conidia examined) for the 3 and 24 h sampling times were, respectively: (a) B2 TI (393, 321); (b) WH1 2.8 (439, 319); (c) WH1 4.1 (430, 321); (d) WH1 9.5 (391, 441); (e) WH2 5.2 (477, 215); (f) WH2 5.3 (461, 250); (g) WH2 11.4 (282, 283); (h) TC1 (499, 451).

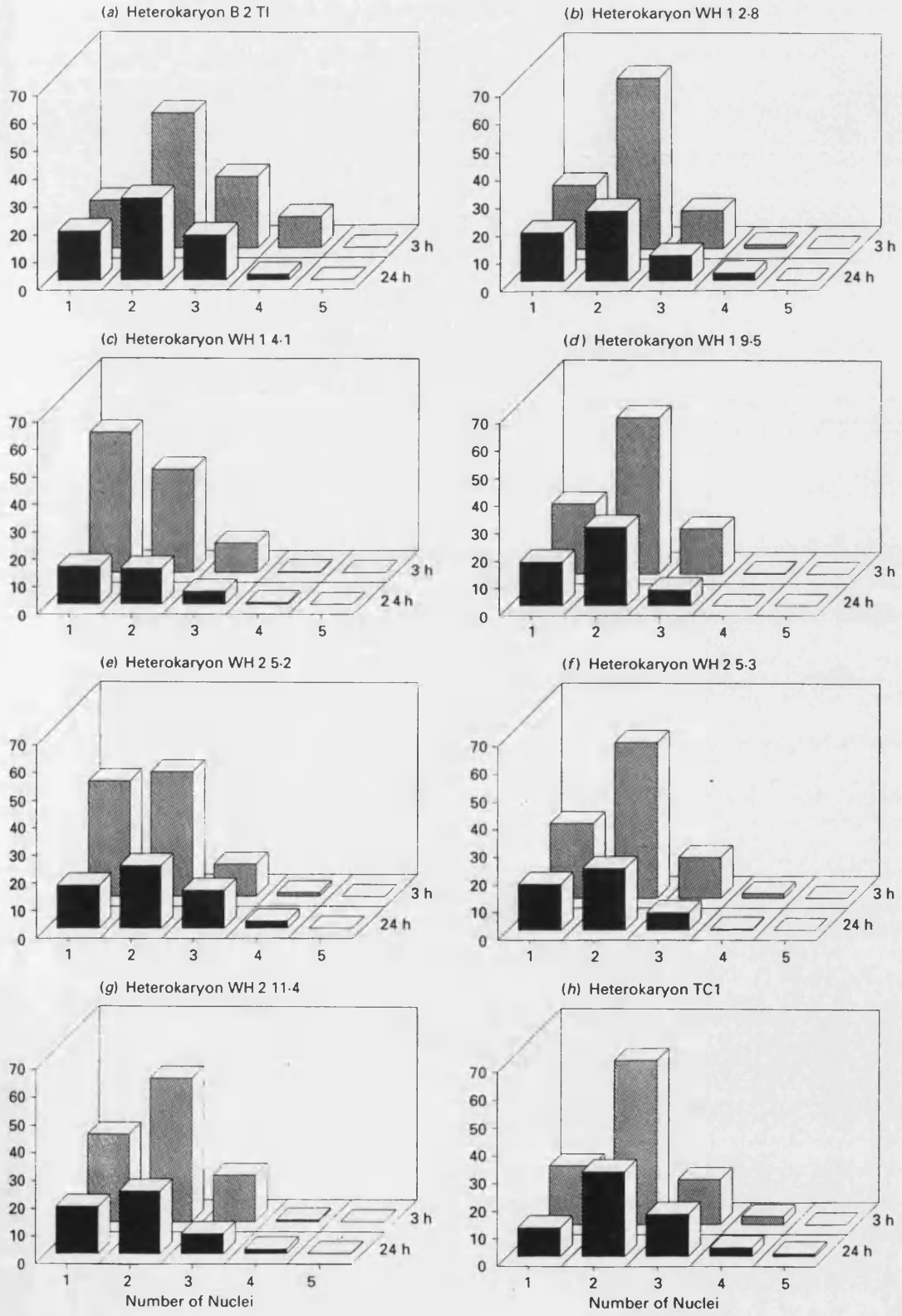


Figure 3.2 continued. Distribution of numbers of nuclei in ungerminated conidia from naturally-occurring heterokaryotic isolates of *H.annosum* at 3 and 24 h after plating out onto MEA at 20°C. Values shown are corrected for overall percentage germination (frequency at any given time x proportion of conidia not germinated). Sample sizes (number of conidia examined) for the 3 and 24 h sampling times were, respectively: (i) Fr 125 (597, 288); (j) Fr 86 (291, 277); (k) Fr 162 (426, 412); (l) Fr 185 (378, 250); (m) Fr 186 (358, 236); (n) Fr 188 (692, 424); (o) Fr 212 (136, 380).

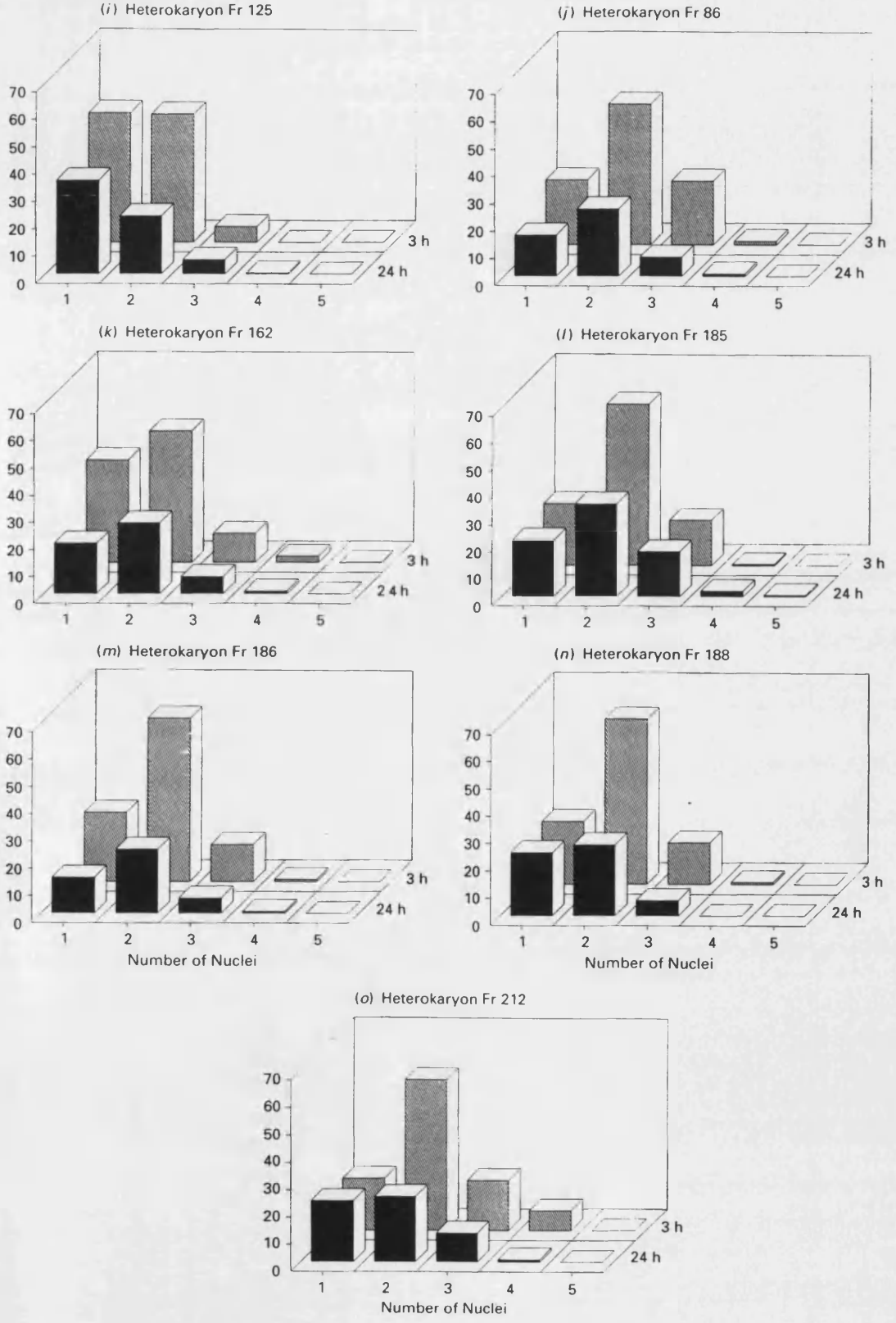


Table 3.4. *Chi-squared, χ^2 (4 df), tests of the hypothesis that nuclear number distributions in ungerminated conidia obtained from naturally-occurring heterokaryons do not vary over time.*

Strain	24 h	Strain	24 h
B2 TI	9.161 nsd	Fr 86	10.04 *
WH1 2·8	23.23 ***	Fr 125	18.30 **
WH1 4·1	9.950 *	Fr 162	1.790 nsd
WH1 9·5	7.503 nsd	Fr 185	17.37 **
WH2 5·2	33.96 ***	Fr 186	3.060 ***
WH2 5·3	9.244 nsd	Fr 188	49.21 ***
WH2 11·4	7.779 nsd	Fr 212	28.34 ***

Comparisons were made between the 3 h and 24 h data. P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different

Table 3.5. *Recovery of homokaryotic and heterokaryotic genotypes via conidia from laboratory-synthesized allopatric heterokaryons of Heterobasidion annosum.*

Strain	Conidia PGM	*n*	%A	%B	χ^2_1	P<	%C	%D	χ^2_2	P<	
FC1	B1·2	10	11	14	35.7	***	15	-	-	-	3
	16·3	60	65	86	-	-	85	-	-	-	-
	Het	22	24	-	-	-	-	1	17.2	***	-
FC2	B1·2	24	30	63	2.63	nsd	61	-	-	-	11
	16·3	14	18	37	-	-	39	-	-	-	-
	Het	41	52	-	-	-	-	20	17.5	***	-
FC3	B3·5	21	33	49	0.02	nsd	48	-	-	-	33
	16·3	22	34	52	-	-	52	-	-	-	-
	Het	21	32	-	-	-	-	5	16.1	***	-
FC4	B3·5	24	43	69	4.83	*	66	-	-	-	39
	16·3	11	20	31	-	-	34	-	-	-	-
	Het	21	38	-	-	-	-	15	7.32	**	-
FC5	B3·6	19	26	43	0.82	nsd	45	-	-	-	24
	16·3	25	35	57	-	-	55	-	-	-	-
	Het	28	39	-	-	-	-	25	3.19	nsd	-
FC6	B3·6	25	40	71	6.43	*	66	-	-	-	33
	16·3	10	16	29	-	-	34	-	-	-	-
	Het	28	44	-	-	-	-	26	4.70	*	-
FC7	B1·2	13	15	32	3.79	nsd	37	-	-	-	12
	34·1	25	29	68	-	-	63	-	-	-	-
	Het	49	56	-	-	-	-	30	12.3	***	-
FC8	B3·5	14	18	23	17.1	***	30	-	-	-	22
	34·1	46	61	77	-	-	70	-	-	-	-
	Het	16	21	-	-	-	-	27	0.74	nsd	-
FC9	B3·6	18	21	32	7.14	***	35	-	-	-	7
	34·1	38	43	68	-	-	65	-	-	-	-
	Het	32	36	-	-	-	-	17	8.43	**	-
FC10	B3·6	10	11	11	53.5	***	18	-	-	-	7
	34·3	79	86	89	-	-	82	-	-	-	-
	Het	3	3	-	-	-	-	19	11.5	***	-
FC11	B3·6	23	26	40	2.12	nsd	42	-	-	-	10
	34·5	34	39	60	-	-	58	-	-	-	-
	Het	31	35	-	-	-	-	19	5.86	*	-
FC12	B3·6	12	14	18	28.5	***	22	-	-	-	12
	12·1	56	65	82	-	-	78	-	-	-	-
	Het	18	21	-	-	-	-	12	2.49	*	-

n, number of conidia isolated; %A, overall genotype percentage; %B, homokaryotic genotype percentage, χ^2_1, chi-squared (1 df) comparing the observed ratios of homokaryotic genotypes with a 1:1 ratio; %C, calculated mycelial nuclear percentage; %D, expected percentage of heterokaryons, χ^2_2, chi-squared (1 df) comparing the observed and expected recovery of heterokaryons; PGM, percentage of germlings that fail to develop following transfer to MEA. P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different.

significant differences were found (F = 2.355; t (22 df) = 2.869 and P<0.001). In contrast to these findings, only one of the naturally-occurring heterokaryons possessed a nuclear ratio that deviated significantly from 1:1 - Table 3.6.

When the final ratios for heterokaryons containing the British genotypes B1.2, B3.5 and B3.6 were compared, a dominance hierarchy was observed. When in combination with nuclei from KK91116.3, and with their own cytoplasmic backgrounds, B3.5 > B3.6 > B1.2 whilst in the KK91116.3 cytoplasm, B3.6 > B3.5 > B1.2. In their own cytoplasmic backgrounds the British sib-related strains (B3.5 and B3.6) fared equally well, and their 'competitive ability' only differed slightly in non-resident cytoplasms (χ^2 = 4.966; P>0.010). When examined in their own cytoplasmic backgrounds but now with nuclei from strain KK89034.1, B3.5 > B3.6 > B1.2. Here, the difference in competitive ability between B3.5 and B3.6 was large but not significantly different. Overall the 'competitive' abilities of the three British genotypes were similar to those described for them earlier in laboratory-synthesized sympatric heterokaryons. When sib-related strains KK87035.3 and KK87035.5 were compared in combination with B3.6 nuclei and the B3.6 cytoplasmic background, (ie heterokaryons FC10 and FC11) the genotype recovery patterns indicated that KK87035.3 > KK87035.5; furthermore this difference was very highly significant (χ^2 = 16.77; P<0.001).

In the majority of cases, given the recovery rates of the component homokaryons from a heterokaryotic mycelium more heterokaryons were recovered from allopatric mycelia than would be expected by chance - Table 3.5. By contrast, recovery rates of heterokaryotic genotypes for naturally-occurring heterokaryons did not generally deviate significantly from that predicted - Table 3.6.

Post-germination mortality

A variable proportion of primary basidiospore germlings derived from naturally-occurring British fruitbodies failed to develop further when transferred to MEA during the initial isolation of some of the strains examined in this study. Post-germination mortality (PGM) ranged from 4% to 50% (mean 24.4 ± 8.1%). A small proportion of germlings (2 to 5%), developed into senescent colonies.

Table 3.6. *Recovery of homokaryotic and heterokaryotic genotypes via conidia from naturally-occurring heterokaryons of Heterobasidion annosum.*

Strain	Conidia PGM	*n*	%A	%B	χ^2_1	P<	%C	%D	χ^2_2	P<	
B2 T	X	52	26	48	0.08	nsd	49	-	-	-	3
	Y	54	29	52	-	-	51	-	-	-	-
	Het	83	45	-	-	-	-	52	2.10	nsd	-
87035	X	58	47	82	15.9	***	68	-	-	-	14
	Y	13	11	18	-	-	32	-	-	-	-
	Het	52	42	-	-	-	-	44	0.07	nsd	-
87034	X32	38	63	1.67	nsd	57	-	-	-	17	
	Y	19	22	37	-	-	43	-	-	-	-
	Het	34	40	-	-	-	-	51	2.13	nsd	-
91112	X	48	39	43	2.03	nsd	46	-	-	-	36
	Y	63	50	57	-	-	54	-	-	-	-
	Het	14	11	-	-	-	-	52	46.6	***	-
91116	X	22	29	50	0.00	nsd	50	-	-	-	22
	Y	22	29	50	-	-	50	-	-	-	-
	Het	32	42	-	-	-	-	52	1.69	nsd	-

n, number of conidia isolated; %A, overall genotype percentage; %B, homokaryotic genotype percentage, χ^2_1, chi-squared (1 df) comparing the observed ratios of homokaryotic genotypes with a 1:1 ratio; %C, calculated mycelial nuclear percentage; %D, expected percentage of heterokaryons, χ^2_2, chi-squared (1 df) comparing the observed and expected recovery of heterokaryons; PGM, percentage of germlings that fail to develop following transfer to MEA. P < ***, 0.001; nsd, not significantly different. X and Y refer to alternative homokaryotic genotypes.

PGM levels varied greatly amongst the conidial germlings derived from allopatric heterokaryons, but the overall mean (17.8 ± 5.1%) did not differ significantly from that observed in the primary basidiospores ($F = 0.220$; t (17 df) = 0.820 and $P <$ nsd). For those heterokaryons that possessed nuclear ratios that deviated significantly from 1:1, PGM dropped as the nuclear ratio asymmetry increased and the number of heterokaryotic conidia recovered decreased.

A sub-set of 108 conidial germlings exhibiting PGM were examined microscopically. Cessation of growth was observed at the following stages: after production of single or double germ-tube(s) (34%); after production of a single branch on the germ-tube(s) (25%); after multiple branching (41%). About a third of the germlings that reached the multiple branching stage produced monilioid hyphae. No anastomoses were evident in any of the failed germlings.

Somato-sexual properties of recovered genotypes

Pairings of recovered strains with their progenitor homokaryons and parental heterokaryons clearly confirmed the identities of the strains in all cases.

The following account is based on a detailed examination of the pairing behaviour of strains recovered from the laboratory-synthesized heterokaryons FC1 and FC2, in which the associated nuclei were the same but the cytoplasms differed. FC1 heterokaryons were morphologically similar to strain B1.2, and FC2 strains were similar to KK91116.3, despite the fact that the nuclear ratios within these heterokaryons were skewed in favour of the invading genotypes. Heterokaryons derived via conidia possessed cultural characteristics identical to those of their progenitor heterokaryons (Figure 3.3). In pairings between heterokaryons derived from single conidia and their synthesized progenitor heterokaryons, the mycelia merged to form uniform mycelial mats. In pairings between synthesized heterokaryons, or between conidium-derived heterokaryons with different cytoplasmic backgrounds the interacting colonies remained phenotypically distinct (Figure 3.4).

When the synthesized progenitor heterokaryons or conidium derived heterokaryons were paired against their parental homokaryons, the mycelia merged to form a single mycelial mat and the homokaryons became phenotypically more like the heterokaryons with which they were

Figure 3.3. Outcome of interactions between strains 1.2 and KK91116.3: (a) original interaction plate between basidiospore derived homokaryons 1.2 and KK91116.3; (b) self paired 1.2; (c) self paired KK91116.3; (d) homokaryon 1.2 derived via conidium from FC1; (e) homokaryon KK91116.3 derived FC1; (f) homokaryon 1.2 derived from FC2 and; (g) homokaryon KK91116.3 derived from FC1.

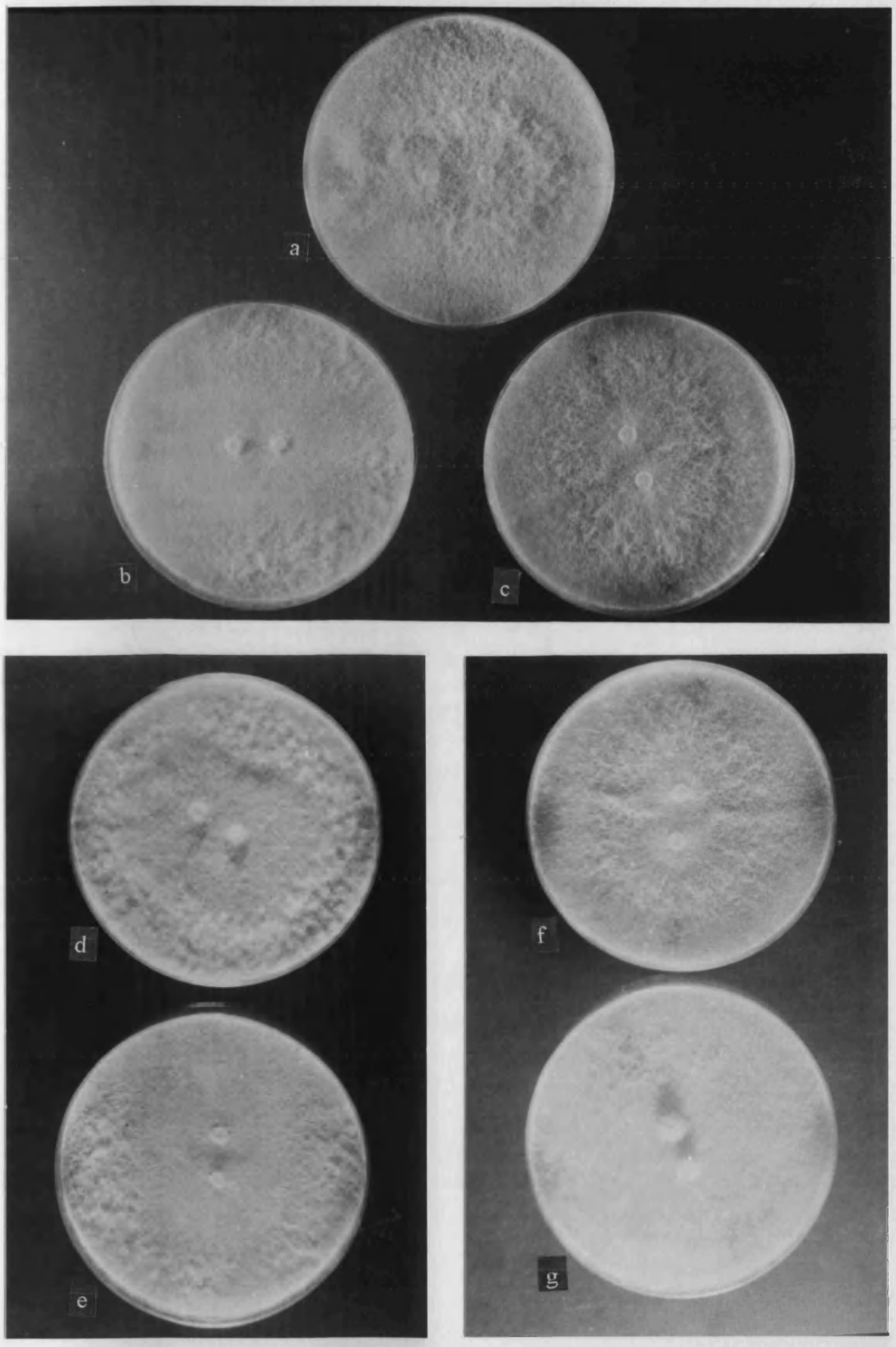
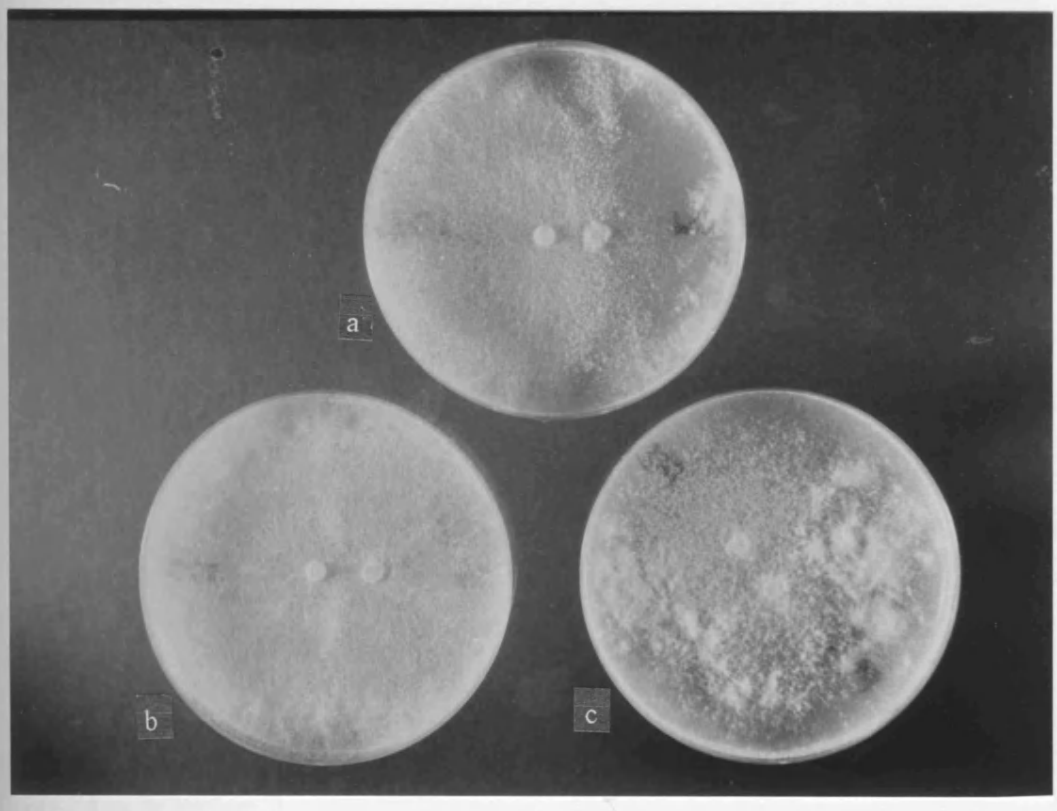


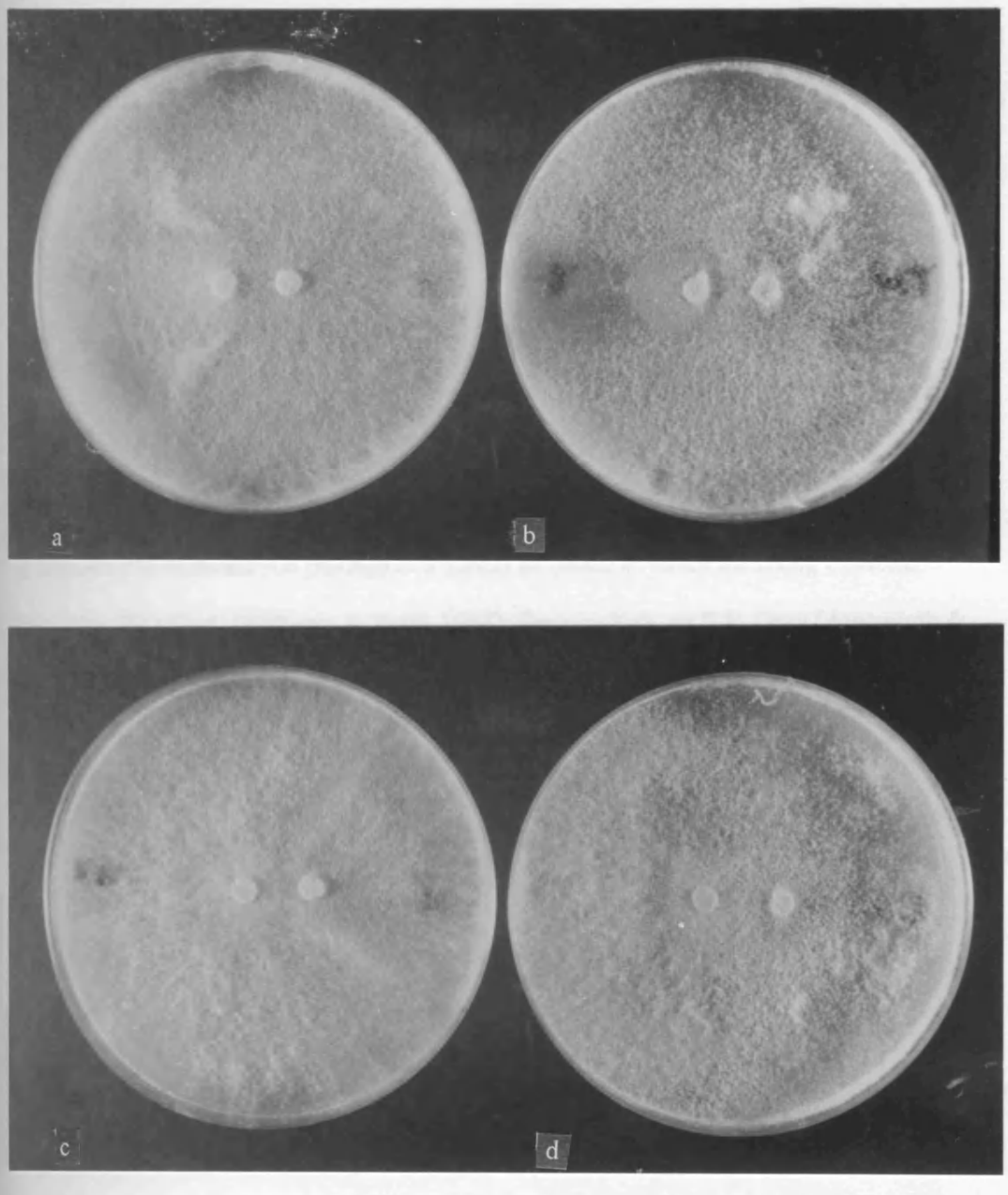
Figure 3.4. Evidence of somatic differentiation between heterokaryons derived from different sides of an interaction zone: (a) pairing between FC1 and FC2; (b) FC1 self pairing and; (c) FC2 self pairing.



paired (Figure 3.5). Similar patterns of interaction were observed when derived homokaryons were paired against derived heterokaryons.

Homokaryons with identical nuclei, but different cytoplasmic partners merged completely, and unlike the heterokaryon pairings there was no evidence of any sustained phenotypic differentiation linked to cytoplasmic background. Pairings between derived homokaryons with different nuclear and cytoplasmic genotypes, or between derived homokaryons and unrelated basidiospore-derived primary homokaryons, led to bilateral heterokaryon formation, accompanied by sparse demarcation zones and distinctive morphological changes. When homokaryons derived from the same heterokaryon were re-paired, heterokaryotic associations were regenerated without the production of a demarcation zone.

Figure 3.5. Outcome of interactions between heterokaryons and homokaryons: (a) 1.2 x FC1; (b) 1.2 x FC2; (c) KK91116.3 x FC1 and; (d) KK91116.3 x FC2.



DISCUSSION

The most striking difference between the present results with laboratory-synthesized allopatric heterokaryons and those obtained previously with synthesized sympatric heterokaryons lay in the enhanced recovery of non-resident relative to resident nuclear genotypes. Although asymmetric nuclear ratios were observed previously, they were largely attributed to differences in the intrinsic rates of proliferation of nuclear genotypes (independent of cytoplasmic context), and to the initial numerical dominance of resident nuclear populations over the invading genotypes. Sympatric homokaryons could therefore be ranked in a strict dominance hierarchy based upon competitive abilities. The considerable numerical dominance of non-resident genomes found in allopatric heterokaryons cannot be explained in the same terms, and implies either the destruction, disabling or dilution of the resident nuclear population following invasion.

Destructive nuclear 'take-over' by non-self nuclei of a recipient mycelium has been described in homokaryon-homokaryon pairings in a variety of basidiomycetes including *Coriolus versicolor* (Fr.) Quel. (Aylmore & Todd, 1984), *Stereum* Pers. ex S.F. Gray (Ainsworth & Rayner, 1989; Ainsworth *et al.*, 1990), and following self-fusions between dikaryons of *Schizophyllum commune* Fr. (Todd & Aylmore, 1985). The possible role of suppression of nuclear division or subjugation of the resident nuclei in generating ratio asymmetry is evident from work by Pittenger & Brawner (1961) who described an allele-specific interaction between strains of *Neurospora crassa* Shear & Dodge. Homokaryotic strains carrying an I allele were able to inhibit the replication of strains with an *i* allele if the proportion of *i* nuclei was less than 70%. Ultimately, this led to the generation of mycelia dominated by I nuclei.

A reduction in viability of resident genomes following non-self invasion could also explain the results. Nuclear genomes that are damaged or altered by invasive nuclei may be supported (or complemented) in a heterokaryon which would then account for the enhanced recovery of heterokaryotic genotypes compared to that expected. Partial or complete loss of genetic information following natural matings has been described in *Stereum hirsutum/ S.complicatum* hybrids (Ainsworth *et al.*, 1992; see later observations on PGM in *Stereum*), and following protoplast fusion between *Penicillium roquefortii* and *P.chrysogenum* (Anné, Eyssen & De

Somer, 1976).

Consistent dominance hierarchies of the type found in laboratory-synthesized sympatric heterokaryons combinations were largely obscured in the laboratory-synthesized allopatric heterokaryons. Nonetheless it was still possible to rank genotypes in the same order when their performance in their own cytoplasmic background against allopatrically derived nuclei was studied. However this was not possible when the relative ability of sib-related nuclei to invade allopatric homokaryons was compared. The competitive equivalence noted previously between sib-related strains in sympatric combinations may not be a general feature of all interactions. Rather, the ability to set up new nucleo-cytoplasmic partnerships may vary amongst strains, and there may be some geographical differentiation of the relevant regulatory loci. An examination of genotype recovery patterns in 'back-crossed' heterokaryons generated between 'derived' homokaryons with novel cytoplasmic partners may give better resolution to this issue (see later).

The differences in behaviour between sympatric and allopatric combinations may be interpreted readily in terms of genomic conflict and co-evolution (Eberhard, 1980; Cosmides & Tooby, 1981; Hurst, 1992; Hurst, Hurst & Johnstone, 1992). In the sympatric situation, co-evolutionary processes may operate to reduce inter-genomic conflict and to minimize take-over. Selection may therefore operate directly against conflicting genomes, removing them from the population, or lead to the emergence of suppressor / modifier systems of attenuation (Dawkins & Krebs, 1979; Hastings, 1992; Hurst, 1992; Law & Hutson, 1992; Hurst & Hamilton, 1992).

Somato-sexual recognition signals (based upon allelic differences or similarities) that function in sympatric situations may have no relevance in an allopatric context. Consequently populations of outcrossing basidiomycetes (and some ascomycetes) might be prone to invasion following geographical transposition of strains, with the attendant scope for destabilization and emergence of novel genotypes. The rapid invasion and replacement of a resident population of *O.ulmi* by *O.novo-ulmi* is thought to have been promoted by introgressive hybridization, in which the hybrids act as a temporary genetic bridge for the *novo-ulmi* clones prior to the elimination of the *ulmi* genotypes from the population (Brasier, 1995).

The main similarity between the results obtained here for allopatrically-derived heterokaryons

and those previously obtained with laboratory-synthesized sympatric heterokaryons was the production of predominantly uninucleate conidia. This pattern of spore production provides a means of escape for the components of a heterokaryon from deleterious heterokaryotic partnerships. The reduced germinability of multinucleate conidia compared to uninucleate conidia also accords with the suggestion that conflict may exist between the nuclei within laboratory-synthesized heterokaryons. The production by naturally-occurring heterokaryons of multinucleate conidia with high germinability and mycelia with 'balanced' nuclear ratios implies that they exhibit less genomic conflict. A situation that has probably arisen from the selection against conflicting combinations of nuclei in natural populations.

Natural populations of *Pleurotus ostreatus* (Jacquin ex Fr.) Kummer contained a deficit of heterozygotes compared to that predicted by the Hardy-Weinburg equilibrium (Vilgalys, 1994). Furthermore, monokaryotic 'bait' strains exhibited selective acceptance of partners when exposed to natural spore rain (Liou, Cowan & Vilgalys, 1994). Such observations may reflect local sampling effects or disequilibrium; alternatively they could represent the outcome of selection against disparity and genomic conflict.

Additional evidence for the existence of conflict between nuclei within heterokaryons is provided by the observed decrease in PGM (post-germination mortality) as the nuclear ratio asymmetries increased. Interactions between non-self nuclei in heterokaryons with highly divergent nuclear ratios occur at a low frequency, thereby reducing the possibility for the expression of conflict or incompatibility.

The remarkable observation that the allopatric heterokaryons often closely resembled the resident parental homokaryons, implies that the phenotype of the heterokaryotic mycelium was independent of the numerically dominant invasive genotypes present. Several explanations are possible for this observation, all of which have important implications for our understanding of genotype-phenotype relationships.

One possible explanation would be that genes in the invasive genomes remained wholly or predominantly unexpressed. Boshart, Nitsch & Schultz (1993) described a phenomenon termed extinction, where cell-type-specific patterns of gene expression, but not general house-keeping gene expression were lost in inter-typic mammalian cell hybrids. Evidence for the non-

expression of non-resident genomes has been found previously for *Stereum hirsutum* nuclei invading *S. complicatum* (Ainsworth *et al.*, 1992). However, for this explanation to hold good, it would be necessary to reject the suggestion made earlier that non-resident genomes support disabled resident genomes in heterokaryons.

Another explanation could be that cytoplasmic factors were responsible for the variations in mycelial phenotype. Some support for this comes from the observation that in many of the laboratory-synthesized and natural heterokaryons, the component genotypes could not be readily identified without recourse to mating studies. However, homokaryons recovered from the FC-series strains with identical nuclei but different cytoplasms were often indistinguishable, implying that their cytoplasm didn't influence phenotype. Furthermore, heterokaryotic conidia were recovered that generated non-parental phenotypes even though the cytoplasm they possessed was identical to that in their progenitor mycelium.

A third possibility is that the processes leading to a particular phenotype become self-sustaining once they have been set in train following an initial genetic input. Usually phenotypic properties are attributed to the presence of genes and gene products, with the environment playing a secondary, modifying role. However in indeterminate systems, there is the potential for autocatalytic processes to re-iterate established patterns of development, in a fashion which once initiated are likely to be extremely sensitive to local environmental conditions. For example, it has recently been suggested that mycelial phenotype may largely be regulated by the action of free radical and active oxygen species at hyphal boundaries (Rayner, Griffith & Wildman, 1994; Rayner 1996). It may therefore be appropriate to attribute some of the fixed organizational properties of a heterokaryotic mycelium to 'hyperepigenetic' inheritance, which once initiated, is largely beyond immediate genetic control (Rayner, Ramsdale & Watkins, 1995).

The reduced level of somatic incompatibility expressed between isolates recovered from a common heterokaryon suggests that some form of cellular memory exists. Heterokaryosis presumably involves mechanisms that limit (override) the expression of incompatibility and ultimately leads to sexual differentiation. Derived homokaryons may be epigenetically imprinted with association-specific information, and so by-pass the initial adjustments needed

to attenuate conflict. Only via the establishment of a genetic *tabula rasa*, perhaps by the passage of a genome through meiosis, will epigenetic instructions be removed.

The retention of heterokaryotic properties following the removal of a genotype from a heterokaryon has been reported previously and includes the continued production of clamp and pseudoclamp cells (Harder, 1926,1927a; Raper & Raper, 1964; Wessels, Hoeksema & Stemerding,1976; Butler,1984; Raper,1985). In some cases these properties were extremely stable and persisted for several hundred nuclear generations.

SECTION IV

ALTERED SOMATO-SEXUAL RECOGNITION RESPONSES AND NUCLEAR NUMBERS IN CONIDIA FROM HOMOKARYONS OF *HETEROBASIDION ANNOSUM* (FR.) BREF. DERIVED MITOTICALLY FROM HETEROKARYONS

INTRODUCTION

In previous sections, the allocation of nuclear numbers to conidia of homokaryons and heterokaryons of *Heterobasidion annosum* has been suggested to reflect the occurrence of inter-nuclear conflicts. Homokaryons (by definition) only produce spores with one type of nucleus, and so they should not be subject to any of the conflicts arising from disparity between non-self genetic entities, or as a result of direct developmental incompatibilities between nuclei. So that advantage can be taken of the greater synthetic capacities associated with higher 'genomic loads', homokaryons might therefore simply trade-off the benefits of producing conidia with high numbers of nuclei per cell against the restrictions imposed by a need for an effective use of resources (law of diminishing returns). Homokaryons would thereby produce conidia that represented the optimal trade-off solution with regard to overall reproductive fitness.

By contrast, since heterokaryons contain different nuclei, they are potentially subject to conflicts. These conflicts create an additional trade-off parameter, since the higher the number of nuclei allocated to a spore, the greater the probability is that it will be heterokaryotic. This may account for the observed tendency for heterokaryons to produce mostly uninucleate conidia which are more germinable than their multinucleate counterparts.

The underlying nature and inheritance of the mechanisms by which heterokaryons limit the effects of genomic conflict, both within mycelia and during conidiogenesis, are therefore of interest. In particular, the degree to which these mechanisms depend solely on the genetic constitution of the interacting nuclear genomes, may become evident from the degree to which the effects persist in homokaryons derived mitotically from heterokaryons in conidia, compared with homokaryons derived meiotically from basidiospores. The following section examines this issue, with respect to patterns of nuclear allocation, spore germination and somato-sexual recognition responses of mitotically and meiotically derived homokaryons.

MATERIALS AND METHODS

Cultural conditions

All isolates, unless otherwise stated, were grown at 20°C in 90 mm diameter unvented Petri dishes containing approximately 15 ml MEA. Stock-cultures were maintained at 4°C on MEA bijou slopes under sterile mineral oil. Designations of the strains examined in this study are provided in Table 4.1

Primary basidiospore pairings

Pairings between four mating-type-compatible homokaryotic strains of *H.annosum* were set up in all possible combinations to generate 'secondary heterokaryons'. Inoculum plugs, 6 mm in diameter, cut from the margins of 7-d-old cultures, were placed 1 cm apart in the centre of MEA plates. Patterns of colony morphogenesis, conidiation, pseudosclerotial plate production (PSP) and demarcation zone formation were followed for 4 weeks.

Twelve hyphal-tip-derived heterokaryons representing all possible primary combinations of nuclear and mitochondrial genotypes used in this investigation were designated as follows; 1·2/3·5 1·2mt, 1·2/3·5 3·5mt, 1·2/3·6 1·2mt, 1·2/3·6 3·6mt, 1·2/6·7 1·2mt, 1·2/6·7 6·7mt, 3·5/3·6 3·5mt, 3·5/3·6 3·6mt, 3·5/6·7 3·5mt, 3·5/6·7 6·7mt, 3·6/6·7 3·6mt and 3·6/6·7 6·7mt.

The morphologies and growth rates of these heterokaryons were also examined following transfer to fresh MEA. Growth rates were determined from measurements of two perpendicular radial dimensions for each of five replicate plates after four and seven days growth at 20°C. After 4 wk growth at 20°C, PSP production was assessed independently by five impartial observers; each observer was requested to rank PSP production (from highest to lowest) for five replicates of each of the twelve heterokaryon combinations - ie 60 plates were ranked. Ranked data were then compared using non-parametric statistical procedures.

Recovery of derived 'secondary' homokaryons

Spore suspensions were prepared from each heterokaryon and spread onto MEA plates at various dilutions. After 24 h incubation at 20°C, single germlings were transferred to Repli dishes (Bibby Sterilin Ltd). After 4 wk, the identities of a sub-set of the single spore isolates were assigned on the basis of their distinctive cultural characteristics and mating-type

Table 4.1. *Designation of Heterobasidion annosum strains examined*

Primary homokaryons	Parents
B1·2	FB1
B3·5	FB3
B3·6	FB3
B6·7	FB6

Secondary heterokaryons	Parents
1·2/3·5 1·2mt	1·2 + 3·5
1·2/3·5 3·5mt	1·2 + 3·5
1·2/3·6 1·2mt	1·2 + 3·6
1·2/3·6 3·6mt	1·2 + 3·6
1·2/6·7 1·2mt	1·2 + 6·7
1·2/6·7 6·7mt	1·2 + 6·7
3·5/3·6 3·5mt	3·5 + 3·6
3·5/3·6 3·6mt	3·5 + 3·6
3·5/6·7 3·5mt	3·5 + 6·7
3·5/6·7 6·7mt	3·5 + 6·7
3·6/6·7 3·6mt	3·6 + 6·7
3·6/6·7 6·7mt	3·6 + 6·7

Secondary homokaryons	Parents
1·2N 1·2mt :1·2/3·5	1·2/3·5 1·2mt
1·2N 1·2mt :1·2/3·6	1·2/3·6 1·2mt
1·2N 1·2mt :1·2/6·7	1·2/6·7 1·2mt
1·2N 3·5mt :1·2/3·5	1·2/3·5 3·5mt
1·2N 3·6mt :1·2/3·6	1·2/3·6 3·6mt
1·2N 6·7mt :1·2/6·7	1·2/6·7 6·7mt
3·5N 3·5mt :1·2/3·5	1·2/3·5 3·5mt
3·5N 3·5mt :3·5/3·6	3·5/3·6 3·5mt
3·5N 3·5mt :3·5/6·7	3·5/6·7 3·5mt
3·5N 1·2mt :1·2/3·5	1·2/3·5 1·2mt
3·5N 3·6mt :3·5/3·6	3·5/3·6 3·6mt
3·5N 6·7mt :3·5/6·7	3·5/6·7 6·7mt
3·6N 3·6mt :1·2/3·6	1·2/3·6 3·6mt
3·6N 3·6mt :3·5/3·6	3·5/3·6 3·6mt
3·6N 3·6mt :3·6/6·7	3·6/6·7 3·6mt
3·6N 1·2mt :1·2/3·6	1·2/3·6 1·2mt
3·6N 3·5mt :3·5/3·6	3·5/3·6 3·5mt
3·6N 6·7mt :3·6/6·7	3·6/6·7 6·7mt
6·7N 6·7mt :1·2/6·7	1·2/6·7 6·7mt
6·7N 6·7mt :3·5/6·7	3·5/6·7 6·7mt
6·7N 6·7mt :3·6/6·7	3·6/6·7 6·7mt
6·7N 1·2mt :1·2/6·7	1·2/6·7 1·2mt
6·7N 3·5mt :3·5/6·7	3·5/6·7 3·5mt
6·7N 3·6mt :3·6/6·7	3·6/6·7 3·6mt

responses. Suitable strains derived from each heterokaryon were also subjected to molecular analyses to confirm their nuclear and mitochondrial genotypes.

Molecular confirmation of strain identity

Mycelia from the four progenitor homokaryons and 24 putative secondary derived homokaryons were grown for 10-d at 20°C on 8 cm diameter, sterile cellulose discs (350 POO, BCL Cellophane) overlying MEA. Mycelium from five plates per sample was harvested, homogenized and freeze-dried overnight. DNA was extracted using a modification of the method of Raeder & Broda (1985). Powdered mycelium was suspended in chilled extraction buffer (200 mM Tris.HCl pH 8.5; 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and agitated for 30 mins. Proteins were removed with an equal volume of phenol / chloroform / iso-amyl alcohol (25:24:1) and then twice more with chloroform / iso-amyl alcohol. DNA was precipitated with 0.6 volumes of isopropanol at -20°C, and recovered by centrifugation at 13 000 rpm for 10 min. DNA pellets were washed twice in 70% ethanol, air-dried and then resuspended in 10 mM Tris.HCl pH 7.5, 1 mM EDTA.

DNA samples were digested according to the manufacturers protocol (NBL) with RNAse A and EcoRV in 50 mM Tris.Cl, 50 mM NaCl, 10 mM $MgCl_2$ buffer. Digested DNA samples were loaded onto 0.7% agarose gels, and separated in 1xTBE buffer for 720 volt hours at room temperature (Sambrook *et al*, 1989). DNA was Southern transferred to Hybond N (Amersham) using neutralizing solution as the mobile phase.

Blotted DNA samples were probed initially with Jeffreys' minisatellite fingerprinting probe 33.15 (Jeffreys, Wilson & Thein, 1985) and then successively with each of the mitochondrial DNAs obtained from the four parental homokaryons. Mitochondrial DNA was purified according to the method of Rogers, Buck & Brasier (1987). Probe DNA was radiolabelled by the method of Feinberg & Vogelstein (1983). Hybridization with Jeffreys' probe was carried out under low stringency conditions, and at high stringency with the mitochondrial DNA probes, following the protocols described by Westneat *et al* (1988).

Twenty-four derived homokaryons were identified for further analysis, one for each of the possible nuclear, mitochondrial and 'background' / past partnership combinations. These strains were designated as follows; 1·2N 1·2mt :1·2/3·5, 1·2N 1·2mt :1·2/3·6, 1·2N 1·2mt

:1·2/6·7, 1·2N 3·5mt :1·2/3·5, 1·2N 3·6mt :1·2/3·6, 1·2N 6·7mt :1·2/6·7, 3·5N 3·5mt :1·2/3·5, 3·5N 3·5mt :3·5/3·6, 3·5N 3·5mt :3·5/6·7, 3·5N 1·2mt :1·2/3·5, 3·5N 3·6mt :3·5/3·6, 3·5N 6·7mt :3·5/6·7, 3·6N 3·6mt :1·2/3·6, 3·6N 3·6mt :3·5/3·6, 3·6N 3·6mt :3·6/6·7, 3·6N 1·2mt :1·2/3·6, 3·6N 3·5mt :3·5/3·6, 3·6N 6·7mt :3·6/6·7, 6·7N 6·7mt :1·2/6·7, 6·7N 6·7mt :3·5/6·7, 6·7N 6·7mt :3·6/6·7, 6·7N 1·2mt :1·2/6·7, 6·7N 3·5mt :3·5/6·7 and 6·7N 3·6mt :3·6/6·7. Where N = nuclear genotype, mt = mitochondrial genotype and, :X/Y = the heterokaryon from which the strain was derived. For comparison purposes the four primary homokaryons were also included in the investigation.

Derived homokaryon pairings

Primary parental basidiospore isolates and the secondary homokaryons derived via conidia from the twelve original heterokaryons were paired against one another in all possible combinations (406 pairings). As with the primary basidiospore pairings described earlier the interactions were examined with respect to morphological changes, patterns of conidiation, pseudosclerotial plate (PSP) production and demarcation zone formation.

Nuclear numbers in conidia

Nuclear numbers in conidia from both primary and secondary homokaryons were examined as described in previous sections. Nuclear numbers in a minimum of 200 ungerminated conidia from 7-d-old homokaryon cultures were assessed for each of two replicates and at five sampling times (3,6,12,24 and 48 h after initial transfer to sterile grid-filters overlaying MEA). Nuclei were visualized by staining air-dried conidia with DAPI (1 $\mu g\ ml^{-1}$). In addition, percentage germination was assessed by counting the germinated and ungerminated conidia in separate random fields of view until a minimum of 500 spores had been examined.

Statistical analyses

Consistency between observers' ranking of PSP production was assessed using Spearmans' rank correlation coefficient (60 df). Friedman's test, and subsequent χ^2 conversions were used to examine the relationships between the strains with respect to PSP production. The Friedman's test is a distribution-free analogue of the normal randomized complete block design of experiment. In this study, observers correspond to blocks, and strains correspond to treatments.

The effects of nuclear genotype, mitochondrial genotype and past history upon the outcome of interactions was assessed by categorizing the pairings into 72 different groups (Table 4.2) and then looking for trends. The groups reflect differences in the relatedness of the strains and their past history. For many attributes it was found that different categories could be merged, with no loss of resolution.

Data for nuclear number distributions in ungerminated conidia were analysed using χ^2 tests corrected for discontinuity when appropriate (Milton & Tsokos, 1983). The data for different replicates were combined since few significant differences were detected between them. The allocation of nuclei to conidia after different incubation times was then compared against the allocation at 3h and 6 h for each strain. Comparisons were also made between strains with the same nuclei but different mitochondrial partners, and between strains with different nuclei but the same past history and mitochondrial partnerships.

Table 4.2. *Pairing categories, codes and number of each category represented (total pairings = 406) for derived homokaryon interaction experiment.*

Code	Virginity code	Nuclear code	Cytoplasm code	Past code	*n*
1	0	0	0	0	16
2	0	0	0	1	8
3	0	0	0	2	8
4	0	0	0	3	8
5	0	0	0	4	32
6	0	0	0	5	8
7	0	0	1	1	7
8	0	0	1	2	4
9	0	0	1	3	4
10	0	0	1	4	16
11	0	0	2	2	4
12	0	0	2	3	1
13	0	0	2	4	6
14	0	0	2	5	3
15	0	0	3	3	3
16	0	0	3	4	8
17	0	0	3	5	5
18	0	1	0	1	8
19	0	1	0	2	4
20	0	1	0	3	4
21	0	1	0	4	16
22	0	1	1	1	1
23	0	1	1	2	2
24	0	1	1	3	2
25	0	1	1	4	8
26	0	1	2	1	3
27	0	1	2	2	2
28	0	1	2	4	3
29	0	1	3	1	2
30	0	1	3	3	2
31	0	1	3	4	4
32	0	2	0	2	4
33	0	2	0	4	8
34	0	2	0	5	4
35	0	2	1	1	2
36	0	2	1	2	2

Virgin; (0) non-virgin homokaryons, (1) one homokaryon virginal, (2) both homokaryons virginal. Nuclei; (0) different, (1) identical, (2) sib-related, (3) similar by descent. Cytoplasm; (0) different, (1) identical, (2) sib-related, (3) similar by descent. Past; (0) no past association, (1) identical past associations, (2) one previous partner identical, the other sib-related, (3) one previous partner identical, the other similar by descent, (4) one past partner similar by descent, the others not related, (5) no past partners in common, but past partners sib-related to each other, and (6) no past history.

Table 4.2 continued. *Pairing categories, codes and number of each category represented (total pairings = 406) for derived homokaryon interaction experiment.*

Code	Virginity code	Nuclear code	Cytoplasm code	Past code	*n*
37	0	2	1	4	4
38	0	2	2	1	2
39	0	2	2	2	2
40	0	2	2	4	4
41	0	2	2	5	2
42	0	2	3	5	2
43	0	3	0	3	4
44	0	3	0	4	8
45	0	3	0	5	4
46	0	3	1	1	1
47	0	3	1	3	2
48	0	3	1	4	4
49	0	3	2	5	2
50	0	3	3	1	3
51	0	3	3	3	2
52	0	3	3	4	4
53	0	3	3	5	2
54	1	0	0	6	32
55	1	0	1	6	8
56	1	0	2	6	4
57	1	0	3	6	4
58	1	1	0	6	8
59	1	1	1	6	12
60	1	1	2	6	2
61	1	1	3	6	1
62	1	2	0	6	4
63	1	2	1	6	2
64	1	2	2	6	6
65	1	2	3	6	0
66	1	3	0	6	4
67	1	3	1	6	2
68	1	3	2	6	0
69	1	3	3	6	7
70	2	0	0	6	4
71	2	2	2	6	1
72	2	3	3	6	1

Virgin; (0) non-virgin homokaryons, (1) one homokaryon virginal, (2) both homokaryons virginal. Nuclei; (0) different, (1) identical, (2) sib-related, (3) similar by descent. Cytoplasm; (0) different, (1) identical, (2) sib-related, (3) similar by descent. Past; (0) no past association, (1) identical past associations, (2) one previous partner identical, the other sib-related, (3) one previous partner identical, the other similar by descent, (4) one past partner similar by descent, the others not related, (5) no past partners in common, but past partners sib-related to each other, and (6) no past history.

RESULTS

Strain characteristics and outcome of pairings

Strains were chosen for further study on the basis of their distinctive cultural characteristics and, according to the ease with which they could readily be identified upon recovery from heterokaryotic associations. Of the four primary parental strains examined, sib-related strains B3.5 and B3.6 showed most similarity. Both produced fast-effuse, abundant loosely aggregated or felty aerial mycelium. Strain B3.6 was pale cream in colour, and possessed a characteristic 'mohair' appearance, occasionally producing brick-red pseudosclerotial plates. Strain B1.2 produced fast-effuse colonies, accompanied by large numbers of conidiophores supported on raised aerial mycelium; colonies were almost white in overall coloration, with a slightly speckled appearance. Mycelia of strain B6.7 generally produced slow-dense mycelia, which were prone to switch to a fast-effuse mode. This strain produced large numbers of conidiophores upon an appressed mycelium, giving the colonies a distinctive farinaceous appearance.

Interactions between these strains were described earlier. The secondary hyphal-tip-derived heterokaryons were all non-parental in appearance, producing dense aerial mycelium, often accompanied by knotty or fascicular hyphal aggregations. All of the heterokaryons produced some pseudosclerotial plates when exposed to light at 20°C. Many strains also produced speckled mycelia and surface watery droplets - both characteristic features of heterokaryotic mycelia of *H.annosum*. Heterokaryons containing genotype B3.5 discoloured the medium a faint yellow. In general, when heterokaryons with identical nuclear partners but different cytoplasmic backgrounds were compared, no specific morphological characteristics could be attributed to the cytoplasmic genotype. However in one instance, significant differences in morphology were evident; strain 3·6/6·7 3·6mt produced large numbers of PSPs and unusual non-sporulating mycelial mounds, whereas strain 3·6/6·7 6·7mt produced small amounts of PSP, and no mounds.

Confirmation of strain identities

The nuclear genotypes of all experimental strains were assessed using Jeffreys' fingerprinting probe 33.15 - see Figure 4.1a. The results indicated the presence of distinctive RFLP markers

Figure 4.1. (a) EcoRV digested total genomic DNA from parental and derived homokaryons of *Heterobasidion annosum* probed with Jeffreys' fingerprinting probe 33.15. Lanes: (1) 1.2N 1.2mt - parental homokaryon; (2) 1.2N 1.2mt :1.2/3.5; (3) 1.2N 1.2mt :1.2/3.6; (4) 1.2N 1.2mt :1.2/6.7; (5) 1.2N 3.5mt :1.2/3.5; (6) 1.2N 3.6mt :1.2/3.6; (7) 1.2N 6.7mt :1.2/6.7; (8) 3.5N 3.5mt - parental homokaryon; (9) 3.5 N 3.5mt :1.2/3.5; (10) 3.5N 3.5mt :3.5/3.6; (11) 3.5N 3.5mt :3.5/6.7; (12) 3.5N 1.2mt :1.2/3.5; (13) 3.5N 3.6mt :3.5/3.6; (14) 3.5N 6.7mt :3.5/6.7; (15) 3.6N 3.6mt - parental homokaryon; (16) 3.6N 3.6mt :1.2/3.6; (17) 3.6N 3.6mt :3.5/3.6; (18) 3.6N 3.6mt :3.6/6.7; (19) 3.6N 1.2mt :1.2/3.6; (20) 3.6N 3.5mt :3.5/3.6; (21) 3.6N 6.7mt :3.6/6.7; (22) 6.7N 6.7mt - parental homokaryon; (23) 6.7N 6.7mt :1.2/6.7; (24) 6.7N 6.7mt :3.5/6.7; (25) 6.7N 6.7mt :3.6/6.7; (26) 6.7N 1.2mt :1.2/6.7; (27) 6.7N 3.5mt :3.5/6.7; (28) 6.7N 3.6mt :3.6/6.7.

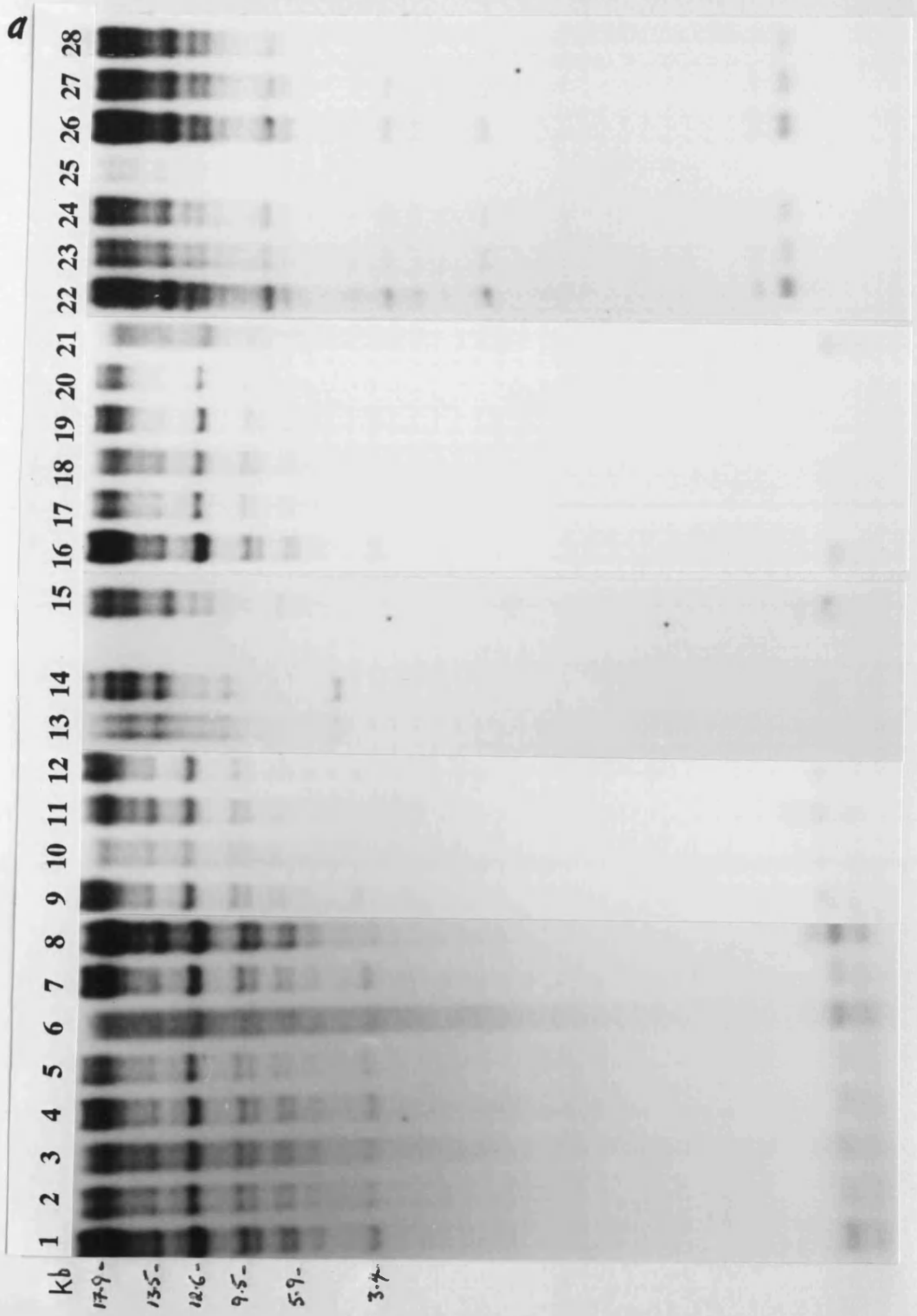


Figure 4.1 continued. (b) EcoRV digested total genomic DNA from parental and derived homokaryons of *Heterobasidion annosum* probed with purified mitochondrial DNA from any of the parental strains. Lanes: (1) 1.2N 1.2mt - parental homokaryon; (2) 1.2N 1.2mt :1.2/3.5; (3) 1.2N 1.2mt :1.2/3.6; (4) 1.2N 1.2mt :1.2/6.7; (5) 1.2N 3.5mt :1.2/3.5; (6) 1.2N 3.6mt :1.2/3.6; (7) 1.2N 6.7mt :1.2/6.7; (8) 3.5N 3.5mt - parental homokaryon; (9) 3.5 N 3.5mt :1.2/3.5; (10) 3.5N 3.5mt :3.5/3.6; (11) 3.5N 3.5mt :3.5/6.7; (12) 3.5N 1.2mt :1.2/3.5; (13) 3.5N 3.6mt :3.5/3.6; (14) 3.5N 6.7mt :3.5/6.7; (15) 3.6N 3.6mt - parental homokaryon; (16) 3.6N 3.6mt :1.2/3.6; (17) 3.6N 3.6mt :3.5/3.6; (18) 3.6N 3.6mt :3.6/6.7; (19) 3.6N 1.2mt :1.2/3.6; (20) 3.6N 3.5mt :3.5/3.6; (21) 3.6N 6.7mt :3.6/6.7; (22) 6.7N 6.7mt - parental homokaryon; (23) 6.7N 6.7mt :1.2/6.7; (24) 6.7N 6.7mt :3.5/6.7; (25) 6.7N 6.7mt :3.6/6.7; (26) 6.7N 1.2mt :1.2/6.7; (27) 6.7N 3.5mt :3.5/6.7; (28) 6.7N 3.6mt :3.6/6.7.

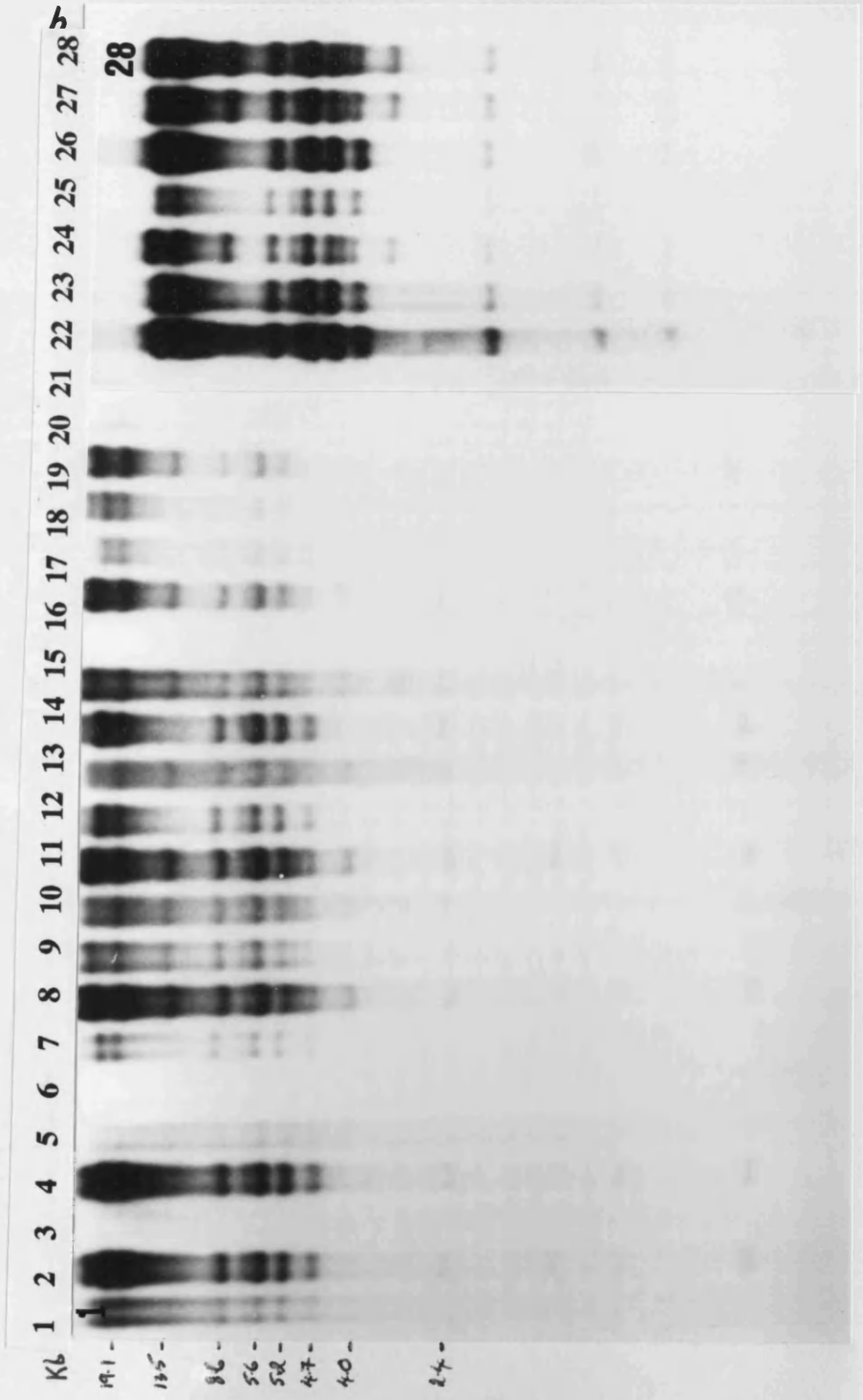
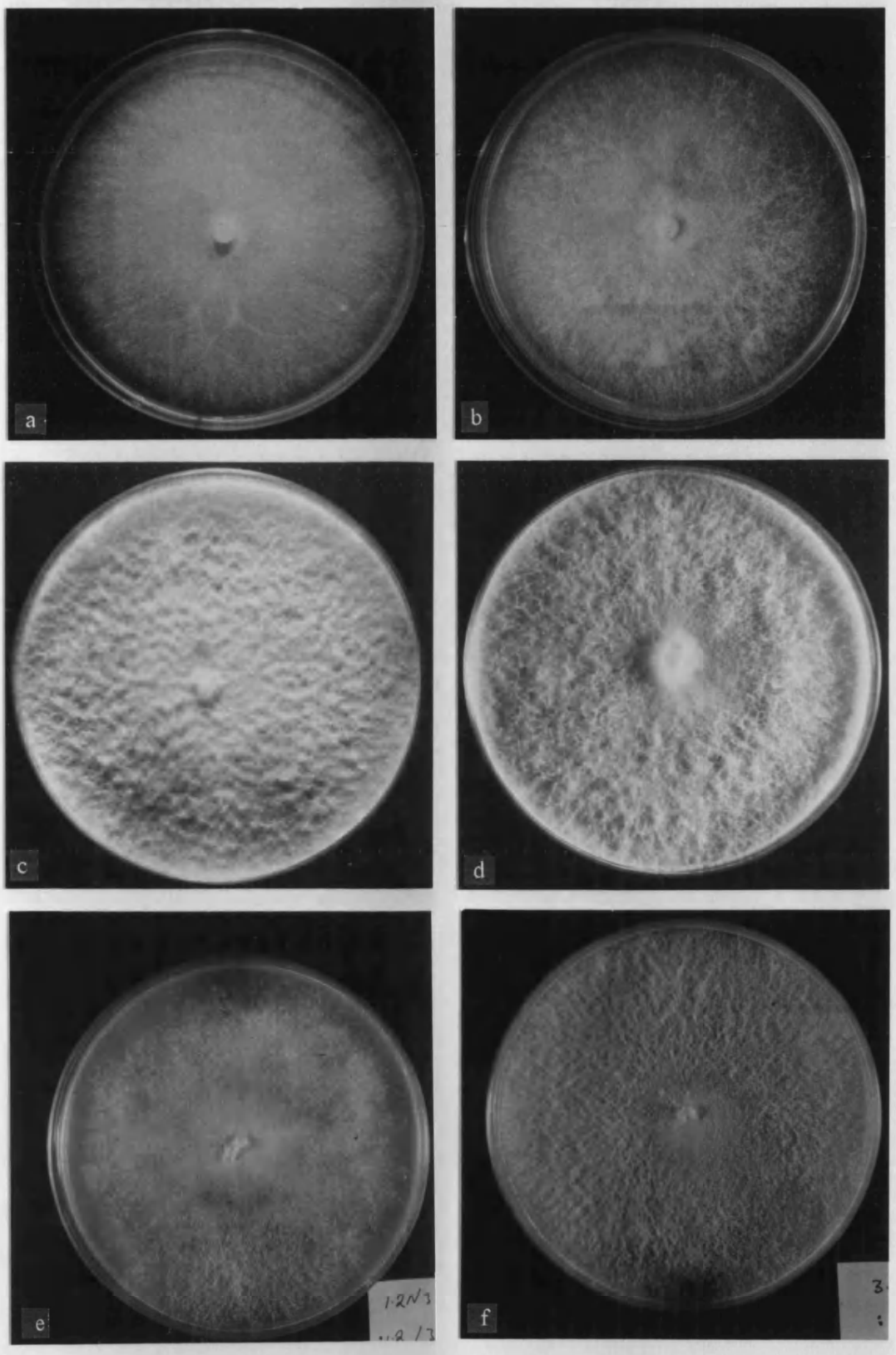


Figure 4.2. Primary basidiospore and derived homokaryotic strains of *Heterobasidion annosum*. (a) Primary homokaryon 1.2; (b) primary homokaryon 3.6; (c) derived homokaryon 1.2N 1.2mt :1.2/3.6; (d) derived homokaryon 3.6N 1.2mt :1.2/3.6; (e) derived homokaryon 1.2N 3.6mt :1.2/3.6; and (f) derived homokaryon 3.6N 3.6mt :1.2/3.6. All plates grown in darkness on MEA for 4 wk.



for each of the parental strains. No genomic alterations or re-arrangements were detected as a result of heterokaryosis, since the marker positions for the component homokaryons remained unchanged following recovery from a heterokaryotic association. Considerable homo-allelism was observed between the sib-related strains B3.5 and B3.6, though some polymorphism in the lowest molecular weight markers enabled them to be distinguished.

Two different mitochondrial haplotype patterns were generated when total genomic DNA, cut with EcoRV, was probed with purified mitochondrial DNA - see Figure 4.1b. The first pattern was shown by the sib-related strains, B3.5 and B3.6. The genome size was estimated to be approximately 113 kb. A second pattern was seen in the two non-sib-related strains B1.2 and B6.7. These strains possessed slightly smaller mitochondrial genomes of around 93 kb. Both types of mitochondrial DNA when used as probe against a range of target samples generated the same patterns.

Derived homokaryon characteristics: general

Secondarily derived homokaryotic strains 1·2N 1·2mt :1·2/3·6, 1·2N 1·2mt :1·2/6·7, 3·5N 3·5mt :3·5/6·7, 3·5N 6·7mt :3·5/6·7, 6·7N 3·6mt :3·6/6·7 and 6·7N 6·7mt :1·2/6·7 were morphologically indistinguishable from their primary homokaryotic progenitors. However most derived strains exhibited some phenotypic changes following their association with other genotypes within a heterokaryon (Figure 4.2). These changes were both morphological and behavioural - ranging from the continued production of certain secondary mycelial characteristics such as speckled mycelia, watery droplets on aerial hyphae and PSPs, to fundamental alterations in somato-sexual behaviour (Table 4.3). In addition, strain 6.7N 3.5mt :3.5/6.7 produced a low number of conidiophores, a feature most uncharacteristic of a B6.7 isolate.

Homokaryons derived from heterokaryons with identical nuclear partners, but different cytoplasms often showed similar patterns of colony development. When such strains were paired with different members of a sib-related-series the interaction outcomes were often very similar regardless of the mitochondrial partner with which they were associated.

Figure 4.3. Synthesized heterokaryotic strains of *Heterobasidion annosum* showing (a) Pseudosclerotial plate or 'grenzhaut' production following exposure to light, and (b) droplet formation on aerial mycelium of a derived homokaryon.

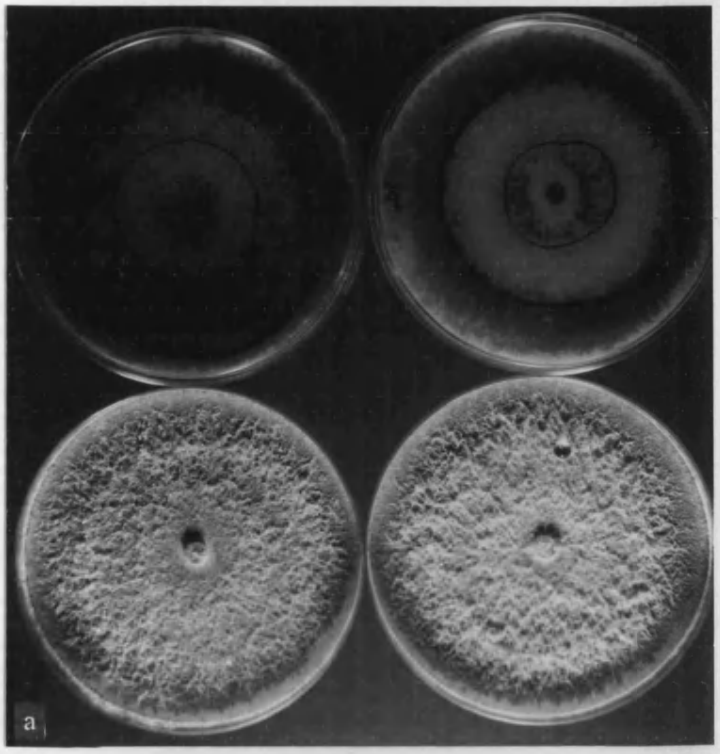
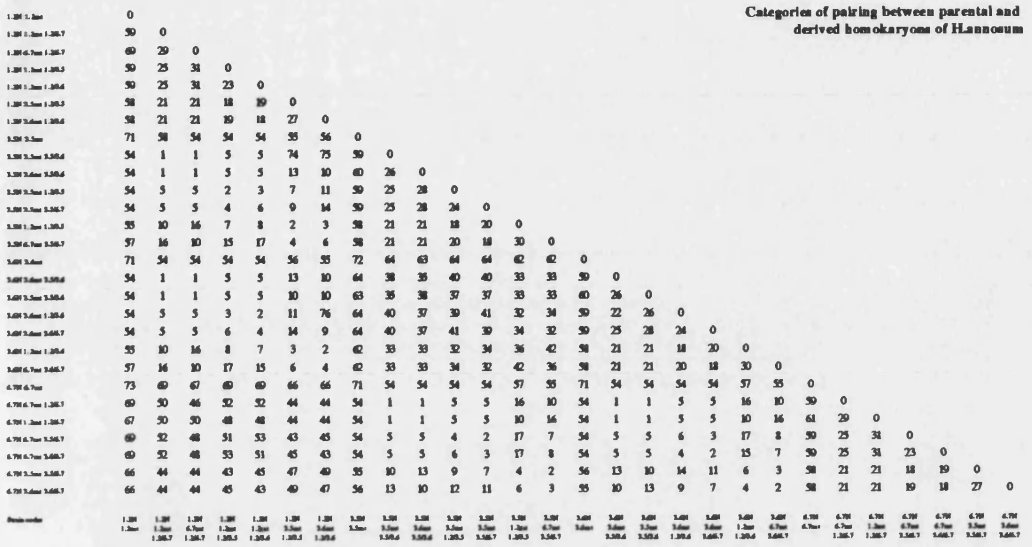
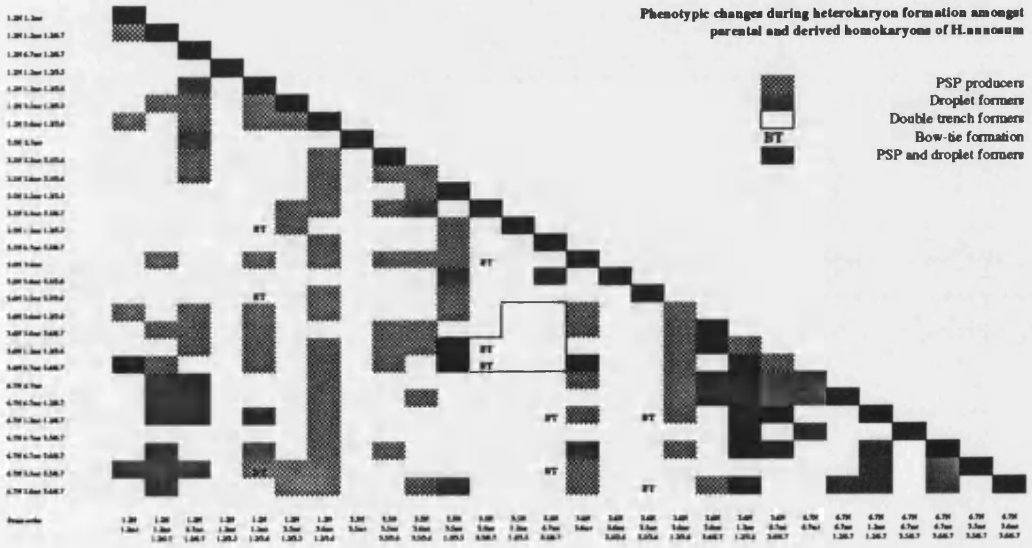
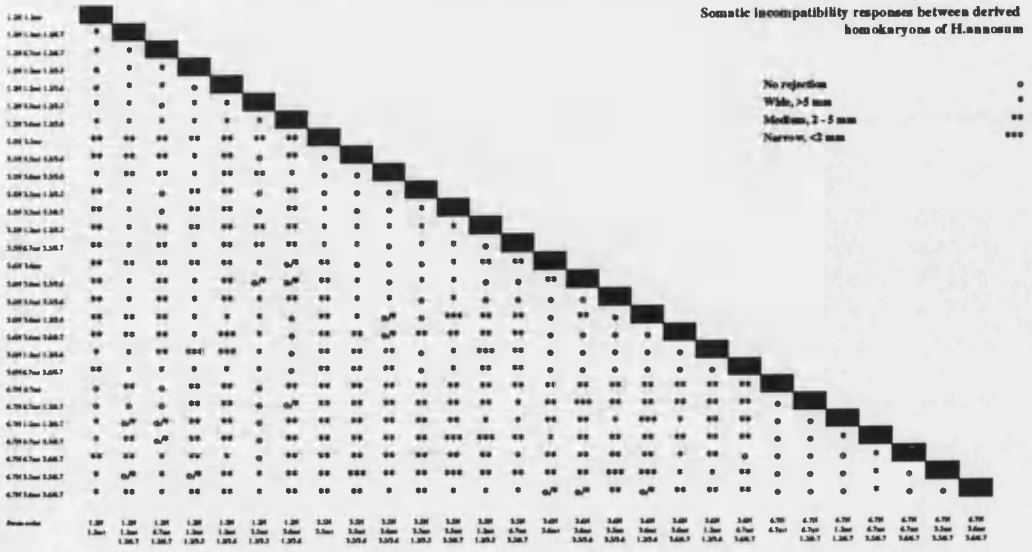


Table 4.3. *Categorization of the 406 pairings between parental and derived homokaryon pairings examined. Codes are the same as those given in Table 4.2.*







Strain characteristics: PSP production

As already described, all of the heterokaryotic isolates produced brick-red pseudosclerotial plates when exposed to light - see Figure 4.3a. The results of the semi-quantitative analysis of PSP production using a panel of impartial observers are presented in Table 4.4. An inspection of the data ranked by individual observers suggests that this approach is both reliable and reproducible. Spearmans' correlation coefficients were all high (Table 4.5).

Friedmans' non-parametric analysis of variation indicated a high level of genotypic differentiation between heterokaryons. The mitochondrial genotype did not influence the level of PSP produced in most strains, but significant differences were detected when the reciprocal heterokaryons of the B1.2 x B6.7 and B3.5 x B3.6 pairings were compared. This was surprising since the mitochondrial genotypes of the homokaryons, and therefore the resulting heterokaryons from these pairings, were shown by molecular analyses to be identical.

Though PSP production is not normally associated with homokaryons in *H.annosum*, nine of the derived homokaryotic strains also produced them. Four of these homokaryons contained B3.6 nuclei and four more were homokaryons that had been associated with B3.6 nuclei and also possessed B3.6 mitochondria. No homokaryotic strains with B3.5 mitochondria (which were genotypically indistinguishable from those of B3.6) produced PSPs. Therefore the production of pseudosclerotial plates is unlikely to be under the direct control of the mitochondrial genome *per se*, but may be acquired either as a result of heritable epigenetic nuclear modifications triggered by interactions with B3.6 nuclei, or as a result of the inheritance of a self-sustaining cytoplasmic state.

Unilateral or bilateral PSP production was observed directly in 99 out of the 406 derived homokaryon pairings. Moreover, 92 of these pairings involved strains that were at some stage associated with genotype B3.6 (63 were B3.6 homokaryons, 23 were non-B3.6 strains with B3.6 mitochondria, and 6 had their 'own' mitochondria, but had been in contact with B3.6 nuclei). Of the pairings that involved B3.6 directly, 14 were 'self-pairings', of which nine produced PSPs bilaterally. Of the remaining PSP-producing pairings that involved B3.6 homokaryons, 57% produced the PSPs on the B3.6 side of the interaction. In a small number of pairings (4/63), the PSPs were restricted to the interaction zone itself.

Table 4.4. *Ranked pseudosclerotial plate production of heterokaryotic mycelia of Heterobasidion annosum (continued overleaf).*

Strain	MR (R_j)	ZRW (R_j)	SJB (R_j)	RTP (R_j)	RJW (R_j)	Totals
1·2/3·5	2	5	2	2	2	165
1·2mt	3	6	3	3	3	
	6 (1)	8 (2)	4 (1)	5 (1)	5 (1)	(1)
	10	9	9	9	6	
	11	13	12	16	12	
1·2/3·5	12	10	8	10	9	369
3·5mt	13	14	14	13	14	
	14 (3)	15 (3)	15 (3)	14 (4)	15 (3)	(3)
	16	16	16	15	16	
	18	20	20	25	17	
1·2/3·6	35	34	34	35	23	1103
1·2mt	44	40	41	42	43	
	46 (9)	45 (9)	46 (9)	47 (9)	47 (9)	(9)
	51	46	52	48	48	
	52	49	53	53	49	
1·2/3·6	43	37	42	43	41	1210
3·6mt	45	43	44	45	45	
	48 (10)	48 (10)	50 (10)	50 (10)	50 (10)	(10)
	50	52	51	51	51	
	56	56	55	57	55	
1·2/6·7	28	28	28	26	32	794
1·2mt	31	29	29	27	33	
	32 (7)	30 (7)	32 (7)	29 (7)	34 (7)	(7)
	33	31	33	31	35	
	37	38	35	34	39	
1·2/6·7	8	2	5	4	4	449
6·7mt	9	7	6	6	10	
	27 (4)	27 (4)	18 (4)	19 (3)	25 (4)	(4)
	29	32	23	20	26	
	30	36	24	21	31	

Numbers in parentheses represent final ordering of genotypes by intensity of PSP production (1) = lowest level, (2) = highest level. Observers, RTP (Becky Prowse); SJB (John Broxholme); RJW (Richard Whiteside); ZRW (Zac Watkins); and, MR (Mark Ramsdale). Friedman's statistic comparing genotypes, S = 3523; χ^2 (11 df) = 54.2 ($P<0.001$).

Table 4.4 continued. *Ranked pseudosclerotial plate production of heterokaryotic mycelia of Heterobasidion annosum.*

Strain	MR (R_j)	ZRW (R_j)	SJB (R_j)	RTP (R_j)	RJW (R_j)	Totals
3·5/3·6	17	17	21	18	18	663
3·5mt	20	18	22	23	20	
	21 (6)	21 (4)	27 (6)	28 (6)	21 (5)	(6)
	34	22	37	36	24	
	38	47	40	37	36	
3·5/3·6	1	1	1	1	1	227
3·6mt	5	3	7	7	7	
	7 (2)	4 (1)	10 (2)	8 (2)	8 (2)	(2)
	15	11	13	12	13	
	19	19	26	17	19	
3·5/6·7	4	12	11	11	11	608
3·5mt	22	23	17	22	22	
	23 (5)	26 (6)	19 (5)	24 (5)	28 (6)	(5)
	24	37	25	33	29	
	36	33	38	40	38	
3·5/6·7	25	24	30	30	27	888
6·7mt	26	25	31	32	30	
	39 (8)	35 (8)	36 (8)	38 (8)	37 (8)	(8)
	40	41	43	39	42	
	41	44	45	44	44	
3·6/6·7	42	42	39	41	40	1314
3·6mt	47	51	56	49	53	
	55 (11)	54 (11)	57 (12)	55 (11)	56 (11)	(11)
	57	57	58	56	57	
	58	58	59	58	59	
3·6/6·7	49	50	47	46	46	1351
6·7mt	53	53	48	52	52	
	55 (12)	55 (12)	49 (11)	54 (12)	54 (12)	(12)
	59	59	54	59	58	
	60	60	60	60	60	

Numbers in parentheses represent final ordering of genotypes by intensity of PSP production (1) = lowest level, (2) = highest level. Observers, RTP (Becky Prowse); SJB (John Broxholme); RJW (Richard Whiteside); ZRW (Zac Watkins); and, MR (Mark Ramsdale). Friedman's statistic comparing genotypes, S = 3523; χ^2 (11 df) = 54.2 ($P<0.001$).

Table 4.5. *Consistency of observer rankings as determined by Spearman's rank correlation coefficient, r_S (58 df).*

Observer	Observer	r_S	P<
MR	ZRW	0.951	***
MR	SJB	0.956	***
MR	RTP	0.957	***
MR	RJW	0.960	***
ZRW	SJB	0.932	***
ZRW	RTP	0.936	***
ZRW	RJW	0.950	***
SJB	RTP	0.960	***
SJB	RJW	0.950	***
RTP	RJW	0.949	***

P < ***, 0.001

Overall, a total of 300 pairings that involved B3.6 directly or indirectly were examined. When these were broken down, 87% of pairings between two B3.6 strains produced PSPs; 51% of pairings with just one B3.6 strain produced PSPs; and 18% of pairings in which at least one partner had been in contact with B3.6 produced PSPs. Remarkably only 7.9% of the pairings that had no past-association whatsoever with B3.6 produced PSPs.

Strain characteristics: droplet formation

A similar breakdown can be made for pairings that produced watery droplets either along the interaction interface itself, or upon the emergent heterokaryotic growth (Figure 4.3b). A total of 51 pairings produced droplets on the interaction plates; 37 of these involved strain B6.7, 14 possessed B6.7 mitochondria, and 4 involved strains that had been in association with B6.7 nuclei. Overall, 80% of the droplets were produced on the B6.7 side of the interaction, or on the side of the interaction that involved strains which had been associated with strain B6.7 in the past.

The frequencies with which droplets were formed within comparable groupings were lower than those observed for PSP production. Out of 300 pairings that involved B6.7 genotypes, 32% of pairings between two B6.7 strains produced droplets; 24% of pairings with just one B6.7 strain produced droplets; and 11% of pairings in which at least one partner had been in contact with B6.7 produced droplets. Of the pairings that had no past-association whatsoever with B6.7, only 6% produced droplets. An inverse relationship was apparent between droplet formation and PSP production. Out of a total of 150 pairings that produced either PSPs or droplets, only 16 produced both.

Genetically, B6.7 mitochondria appeared to be indistinguishable from B1.2 mitochondria, so a direct association between droplet formation and mitochondrial background *per se* can be eliminated. The possible involvement of other sources of variation within the cytoplasms of these non-sib-related strains cannot be ruled out.

Strain characteristics: somatic incompatibility responses

Somatic rejection responses between homokaryotic strains of *H.annosum* were expressed as wide > 5mm (*), medium 2 - 5mm (**), or more unusually, narrow < 2mm (***) demarcation

zones of sparse aerial growth along interfacial boundaries (Figure 4.4). In a limited number of pairings, sienna to brown discoloration developed in the medium beneath these zones. The responses for individual pairings are presented in Table 4.6.

When the different components of the interaction categories outlined in Table 4.2 were examined in detail, some important trends became apparent (Table 4.6). Interactions between two 'virgin' homokaryons produced narrower rejection zones (mean 1.67) than interactions between non-virgin homokaryons (mean 1.27) or a virgin strain and a non-virgin strain (mean 1.19). Pairings between strains with identical nuclei rarely exhibited any evidence of somatic incompatibility (mean 0.44).

Interactions between sibs, or strains similar by descent (ie B1.2 or B6.7), produced wide interaction zones. Unrelated nuclear combinations generally produced narrower rejection zones (mean 1.64) with a mean value similar to those for interactions between virgins. No conclusive patterns were apparent when strains with different cytoplasms and different nuclei were paired, but interactions between strains with unrelated cytoplasms did produce the narrowest, or most intense rejection responses. When strains with identical nuclei and different cytoplasmic backgrounds were paired, weak rejection responses were occasionally observed. The least intense rejection responses were observed between strains with cytoplasms that were identical.

With regard to past history, interactions between isolates that had no past partners in common, or that had no past associations whatsoever, produced narrow incompatibility zones. Interactions between strains that were derived directly from the same heterokaryon or between strains in which one past partner was similar by descent, and the other was unrelated produced the weakest rejection responses (means 1.09 and 1.02 respectively).

Strain characteristics: Bow-ties and complex interfacial zones

'Bow-tie interactions', characterized by the production of a flattened, lysed mycelium, widest at the edges of interfacial boundaries between opposing strains, were observed in six pairings between derived homokaryons (Figure 4.5). Unlike similar bow-tie responses in *Stereum hirsutum* (Coates & Rayner, 1985a), watery droplets were only observed along the invasion front in one of these pairings. All bow-tie producing combinations involved strain B6.7.

Table 4.6. *Analysis of somatic incompatibility responses between derived homokaryotic isolates of Heterobasidion annosum.*

Category	o	*	**	***	Mean(1)
0XXX	70	90	125	13	1.27
1XXX	22	19	37	0	1.19
2XXX	1	0	5	0	1.67
X0XX	15	53	119	12	1.64
X1XX	51	31	3	0	0.44
X2XX	12	12	24	1	1.28
X3XX	10	10	19	0	1.23
XX0X	41	51	98	5	1.34
XX1X	28	29	22	4	1.02
XX2X	13	14	21	2	1.24
XX3X	11	15	26	2	1.35
XXX0	0	3	11	2	1.94
XXX1	16	21	15	3	1.09
XXX2	6	12	13	1	1.28
XXX3	9	8	18	1	1.31
XXX4	35	39	32	3	1.02
XXX5	4	7	18	3	1.62
XXX6	23	19	60	0	1.36

(1) Arithmetic mean score for a given class when o = zero value, * = 1, ** = 2 and *** = 3. Category codes refer to; First position - virgin, (0) non-virgin homokaryons; (1) one homokaryon virginal; (2) both homokaryons virginal. Second position - nuclei, (0) different; (1) identical; (2) sib-related; (3) similar by descent. Third position - cytoplasm, (0) different; (1) identical; (2) sib-related; (3) similar by descent. Fourth position - past, (0) no past association; (1) identical past associations; (2) one previous partner identical, the other sib-related; (3) one previous partner identical, the other similar by descent; (4) one past partner similar by descent, the others not related; (5) no past partners in common, but past partners sib-related to each other; and (6) no past history.

Figure 4.4. Somatic incompatibility or 'gap' reactions between homokaryotic strains of *Heterobasidion annosum*. (a,b) Strong somatic incompatibility between primary homokaryotic strains; (c-f) weak or absent incompatibility responses between derived homokaryons.

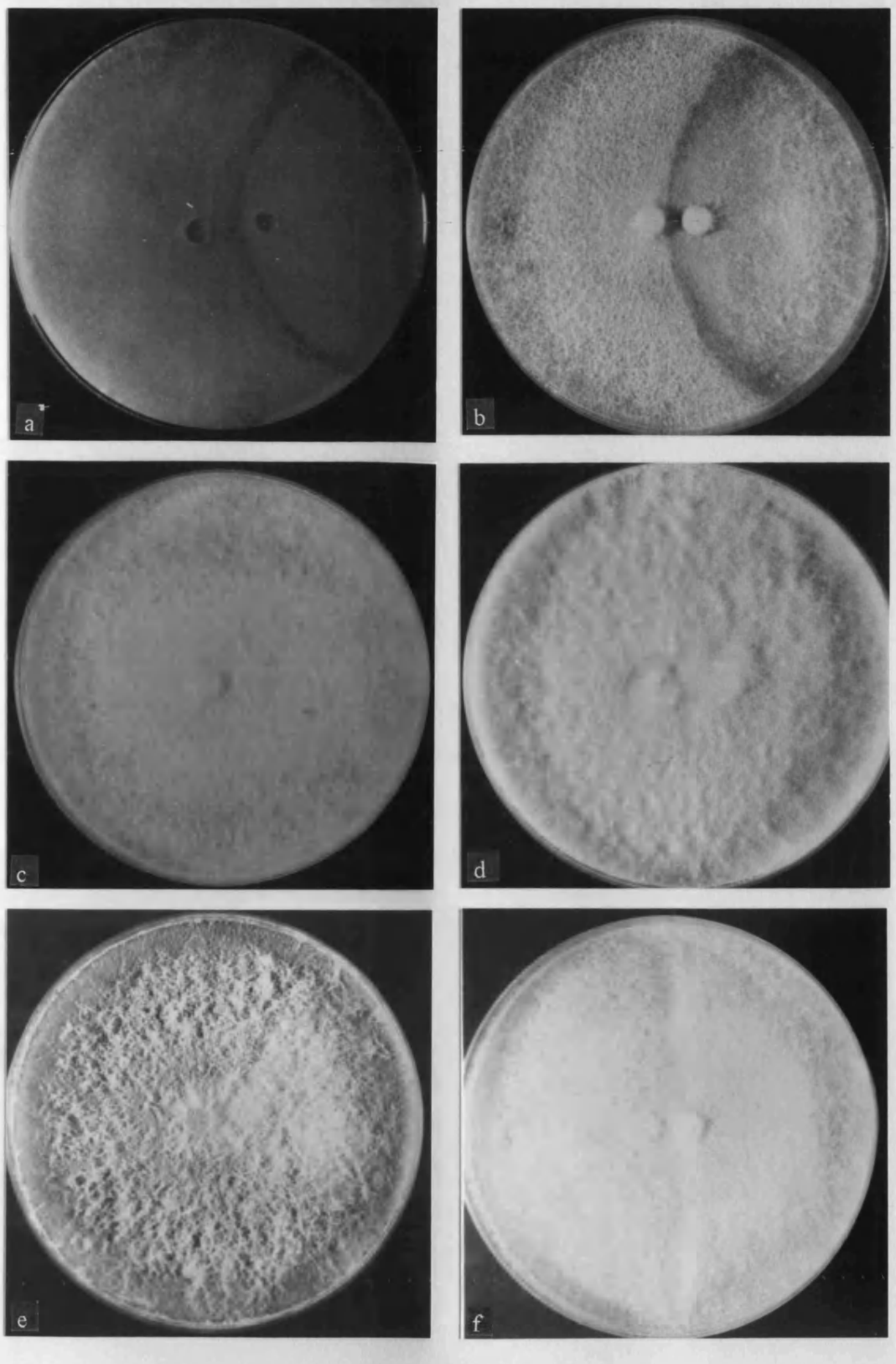


Figure 4.5. Bow-tie shaped somatic incomaptibility zone between two derived homokaryons of *H.annosum*.

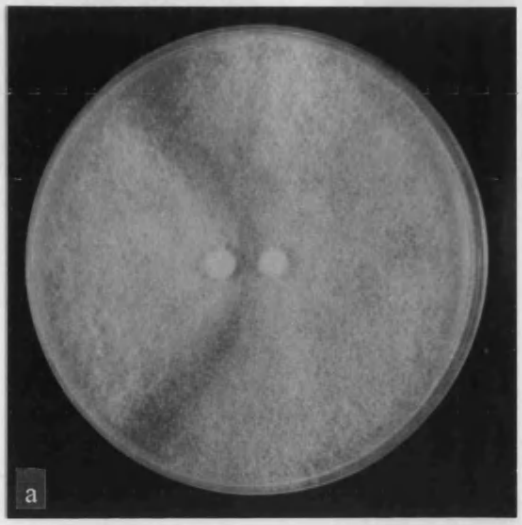
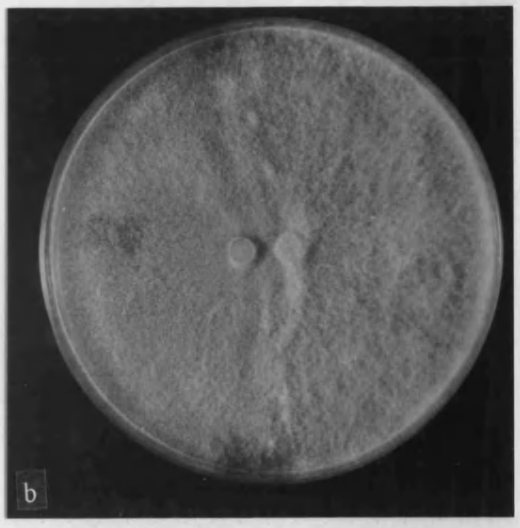


Figure 4.6. Double trench formed between two derived strains of *H.annosum*.



Beyond this, no common theme was evident amongst those strains that produced this pattern of colony development.

A novel type of interaction was also observed in some pairings that resulted in the formation of a double gap zone (Figure 4.6). The contact zone of the interacting strains supported a narrow dense band of aerial, prolifically sporulating mycelium, which was separated on either side by gap zones which were similar to those seen in normal somatic incompatibility responses. This outcome was only observed when the two interacting strains were sib-related (B3.5 with B3.6) and when they had both been associated with the same non-sib related strain in the non-sib related cytoplasmic background.

Nuclear numbers in conidia

The distribution patterns of numbers of nuclei in conidia from derived and naturally-occurring homokaryons are presented in Figure 4.7 and 4.8. Table 4.8 indicates that the distributions varied little between replicates. For most of the isolates examined, the distributions of nuclear number in ungerminated conidia changed significantly over time (see Table 4.9a,b).

Initial nuclear number distribution patterns in derived homokaryotic conidia varied both amongst and between strains (Table 4.10). Derived B3.6 strains produced conidia that were mostly uninucleate, including those derived from the sib-related heterokaryons 3·5/3·6 3·5mt and 3·5/3·6 3·6mt. Strain B3.5 produced mostly uninucleate conidia when derived from heterokaryons involving genotypes B3.6 and B6.7, but predominantly binucleate conidia when taken from heterokaryotic association with genotype B1.2. Strain B1.2 produced mostly uninucleate conidia when recovered from heterokaryotic associations with a B1.2 cytoplasmic background and binucleate conidial distributions when the cytoplasmic background belonged to the other partner. All B6.7-derived homokaryons produced conidia that were primarily binucleate. When the nuclear number distributions of different genotypes from the same heterokaryotic partnership were compared, only 3.6N 3.6mt :3.6/6.7 with 6.7N 3.6mt: 3.6/6.7 and the homokaryons derived from the sib-related heterokaryon 3.5/3.6 3.6mt were statistically homogeneous. All other comparisons were statistically different (Table 4.10). Greater similarity between strains was detected when strains with identical nuclei but different cytoplasms were compared.

Table 4.8. *Chi-squared, χ^2 (4 df), tests of the hypothesis that no differences in nuclear number distribution occur between replicates for each incubation time / genotype for derived homokaryotic strains.*

Strain	3h	6h	12h	24h	48h
1·2N 1·2mt	2.704	3.075	-	1.768	2.374
:1·2/3·5	nsd	nsd		nsd	nsd
1·2N 1·2mt	2.374	4.171	6.748	0.078	1.517
:1·2/3.6	nsd	nsd	nsd	nsd	nsd
1·2N 1·2mt	4.629	3.439	4.226	5.474	-
:1·2/6·7	nsd	nsd	nsd	nsd	
1·2N 3·5mt	1.133	1.263	5.353	0.722	0.883
:1·2/3·	nsd	nsd	nsd	nsd	nsd
1·2N 3·6mt	178	8.435	0.341	3.503	1.197
:1·2/3·	nsd	nsd	nsd	nsd	nsd
1·2N 6·7mt	085	10.14	13.26	0.254	0.074
:1·2/6·	nsd	*	*	nsd	nsd
3·5N 3·5mt	164	3.259	1.018	1.211	-
:1·2/3·	nsd	nsd	nsd		nsd
3·5N 3·5mt	14.20	2.606	2.932	1.222	-
:3·5/3·6	*	nsd	nsd	nsd	
3·5N 3·5mt	2.763	0.220	2.264	3.793	-
:3·5/6·7	nsd	nsd	nsd	nsd	
3·5N 1·2mt	24.71	1.365	0.413	3.482	-
:1.2/3·5	*	nsd	nsd	nsd	
3·5N 3·6mt	3.504	1.425	2.556	3.514	-
:3·5/3·6	nsd	nsd	nsd	nsd	
3·5N 6·7mt	5.509	5.752	1.498	0.701	-
:3·5/6·7	nsd	nsd	nsd	nsd	
3·6N 3·6mt	0.907	4.852	1.823	2.685	-
:1·2/3·6	nsd	nsd	nsd	nsd	
3·6N 3·6mt	6.211	3.677	2.204	2.359	-
:3·5/3·6	nsd	nsd	nsd	nsd	
3·6N 3·6mt	0.541	6.194	0.272	0.065	-
:3·6/6·7	nsd	nsd	nsd	nsd	
3·6N 1·2mt	1.437	4.179	3.995	0.383	-
:1·2/3·6	nsd	nsd	nsd	nsd	
3·6N 3·5mt	1.783	3.451	1.338	-	-
:3·5/3·6	nsd	nsd	nsd		
3·6N 6·7mt	1.129	3.601	0.351	0.869	-
:3·6/6·7	nsd	nsd	nsd	nsd	
6·7N 6·7mt	3.305	1.284	1.395	-	-
:1·2/6·7	nsd	nsd	nsd		
6·7N 6·7mt	4.747	4.505	1.667	-	-
:3·5/6·7	nsd	nsd	nsd		
6·7N 6·7mt	2.155	2.135	0.089	-	-
:3·6/6·7	nsd	nsd	nsd		
6·7N 1·2mt	4.149	6.108	0.237	-	-
:1·2/6·7	nsd	nsd	nsd		
6·7N 3·5mt	6.885	0.918	1.955	-	-
:3·5/6·7	nsd	nsd	nsd		
6·7N 3·6mt	10.44	1.237	(1)	-	-
:3·6/6·7	*	nsd			

P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different. (-) = 100% germination

Table 4.9a. *Chi-squared, χ^2 (4 df), tests of the hypothesis that nuclear number distributions in ungerminated conidia obtained from derived homokaryons do not vary over time. Comparisons were made for combined replicate data against the initial frequencies at 3 h.*

Strain	3h	6h	12h	24h	48h
1·2N 1·2mt	0	3.971	4.735	21.41	-
:1·2/3·5	nsd	nsd	nsd	***	
1·2N 1·2mt	0	13.22	15.14	70.87	11.92
:1·2/3·6	nsd	*	**	***	*
1·2N 1·2mt	0	13.28	5.562	3.852	-
:1·2/6·7	nsd	*	nsd	nsd	
1·2N 3·5mt	0	5.253	18.17	25.95	56.82
:1·2/3·5	nsd	nsd	**	***	***
1·2N 3·6mt	0	3.845	16.79	41.70	19.66
:1·2/3·6	nsd	nsd	**	***	***
1·2N 6·7mt	0	2.836	13.09	38.40	22.57
:1·2/6·7	nsd	nsd	*	***	***
3·5N 3·5mt	0	47.10	55.79	86.43	-
:1·2/3·5	nsd	***	***	***	
3·5N 3·5mt	0	7.263	13.06	38.78	21.15
:3·5/3·6	nsd	nsd	*	***	***
3·5N 3·5mt	0	7.801	18.59	57.05	-
:3·5/6·7	nsd	nsd	***	***	
3·5N 1·2mt	0	20.54	73.26	83.61	-
:1·2/3·5	nsd	***	***	***	
3·5N 3·6mt	0	4.498	7.477	30.39	-
:3·5/3·6	nsd	nsd	nsd	***	
3·5N 6·7mt	0	7.633	26.16	56.54	-
:3·5/6·7	nsd	nsd	***	***	
3·6N 3·6mt	0	2.789	14.82	13.96	-
:1·2/3·6	nsd	nsd	**	**	
3·6N 3·6mt	0	3.135	27.22	45.04	-
:3·5/3·6	nsd	nsd	***	***	
3·6N 3·6mt	0	1.509	61.20	35.95	-
:3·6/6·7	nsd	nsd	***	***	
3·6N 1·2mt	0	3.581	19.63	77.15	-
:1·2/3·6	nsd	nsd	***	***	
3·6N 3·5mt	0	3.541	36.54	-	-
:3·5/3·6	nsd	nsd	***		
3·6N 6·7mt	0	1.322	59.42	114.9	-
:3·6/6·7	nsd	nsd	***	***	
6·7N 6·7mt	0	0.135	32.09	-	-
:1·2/6·7	nsd	nsd	***		
6·7N 6·7mt	0	12.39	21.39	-	-
:3·5/6·7	nsd	*	***		
6·7N 6·7mt	0	2.775	46.09	-	-
:3·6/6·7	nsd	nsd	***		
6·7N 1·2mt	0	13.76	57.49	-	-
:1·2/6·7	nsd	**	***		
6·7N 3·5mt	0	15.63	93.77	-	-
:3·5/6·7	nsd	**	***		
6·7N 3·6mt	0	5.477	14.79	-	-
:3·6/6·7	nsd	nsd	**		

P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different. (-) = 100% germination

Table 4.9b. *Chi-squared, χ^2 (4 df), tests of the hypothesis that nuclear number distributions in ungerminated conidia obtained from derived homokaryons do not vary over time. Comparisons were made for combined replicate data against the initial frequencies at 6 h.*

Strain	3h	6h	12h	24h	48h
1·2N 1·2mt	3.971	0	3.321	10.264	-
:1·2/3·5	nsd	nsd	nsd	*	
1·2N 1·2mt	13.22	0	2.269	31.03	1.965
:1·2/3·6	*	nsd	nsd	***	nsd
1·2N 1·2mt	13.28	0	4.707	12.88	-
:1·2/6·7	**	nsd	nsd	*	
1·2N 3·5mt	5.253	0	7.519	13.70	32.53
:1·2/3·5	nsd	nsd	nsd	**	***
1·2N 3·6mt	3.845	0	13.85	36.29	18.24
:1·2/3·6	nsd	nsd	**	***	**
1·2N 6·7mt	2.836	0	15.43	42.32	24.75
:1·2/6·7	nsd	nsd	**	***	***
3·5N 3·5mt	47.09	0	17.99	25.36	-
:1·2/3·5	***	nsd	**	***	
3·5N 3·5mt	7.263	0	12.85	46.51	22.42
:3·5/3·6	nsd	nsd	*	***	***
3·5N 3·5mt	7.801	0	26.90	66.84	-
:3·5/6·7	nsd	nsd	***	***	
3·5N 1·2mt	20.54	0	65.34	84.33	-
:1·2/3·5	***	nsd	***	***	
3·5N 3·6mt	4.498	0	13.07	44.43	-
:3·5/3·6	nsd	nsd	*	***	
3·5N 6·7mt	7.633	0	15.55	42.66	-
:3·5/6·7	nsd	nsd	**	***	
3·6N 3·6mt	2.789	0	18.16	17.97	-
:1·2/3·6	nsd	nsd	**	**	
3·6N 3·6mt	3.135	0	30.07	48.16	-
:3·5/3·6	nsd	nsd	***	***	
3·6N 3·6mt	1.509	0	65.189	36.82	-
:3·6/6·7	nsd	nsd	***	***	
3·6N 1·2mt	3.581	0	25.49	82.86	-
:1·2/3·6	nsd	nsd	***	***	
3·6N 3·5mt	3.541	0	39.15	-	-
:3·5/3·6	nsd	nsd	***		
3·6N 6·7mt	1.322	0	53.87	108.8	-
:3·6/6·7	nsd	nsd	***	***	
6·7N 6·7mt	0.135	0	29.14	-	-
:1·2/6·7	nsd	nsd	***		
6·7N 6·7mt	12.39	0	15.88	-	-
:3·5/6·7	*	nsd	**		
6·7N 6·7mt	2.775	0	42.70	-	-
:3·6/6·7	nsd	nsd	***		
6·7N 1·2mt	13.76	0	61.91	-	-
:1·2/6·7	**	nsd	***		
6·7N 3·5mt	15.63	0	99.98	-	-
:3·5/6·7	**	nsd	***		
6·7N 3·6mt	5.477	0	19.64	-	-
:3·6/6·7	nsd	nsd	***		

P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different. (-) = 100% germination.

Table 4.9c. *Chi-squared, χ^2 (4 df), tests of the hypothesis that nuclear number distributions in ungerminated conidia obtained from naturally-occurring homokaryons do not vary over time. Comparisons were made between the 3 h and 24 h sampling times.*

Strain	χ^2	Strain	χ^2
B2·1	3.334 nsd	Faf 4·4	7.984 nsd
B2·2	19.67 ***	Faf 8·5	38.57 ***
B2·3	18.57 ***	TC 1	9.246 nsd
B2·4	37.10 ***	TC 32·1	8.111 nsd
B2·5	52.98 ***	TC 111·4	4.488 nsd
B2·6	49.33 ***	TC 122·11	2.437 nsd
B2·7	14.06 **	TC 122·12	28.93 ***
B2·8	8.892 nsd	FaO1	57.14 ***

P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different

Table 4.10. *Examination of variation in allocation of nuclei to conidia in derived homokaryons of Heterobasidion annosum*

Variability within genotypes (nuclei identical)				
1·2Ns	**3·5Ns**	**3·6Ns**		**6·7Ns**
χ^2=396.45 (20 df) ***	χ^2=166.735 (20 df) ***	χ^2=109.34 (20df) ***		χ^2=62.67 (20 df) ***
Major contributors to variation				
1·2N 1·2:12/35	3·5N 3·5:12/35	3·6N 3·6:36/67		6·7N 3·6:36/67
1·2N 1·2:12/67	3·5N 3·5:35/36	3·6N 3·6:12/36		6·7N 6·7:35/67
Minor contributors to variation				
1·2N 1·2:12/36	3·5N 3·5:35/67	3·6N 3·5:35/36		6·7N 3·5:35/67
1·2N 3·5:12/35	3·5N 3·6:35/36	3·6N 3·6:35/36		6·7N 6·7:36/67
Comparisons of different genotypes derived from the same background				
1·2N 1·2:12/35	3·5N 1·2:12/35	67.15	(4 df)	***
1·2N 3·5:12/35	3·5N 3·5:12/35	99.21	(4 df)	***
1·2N 1·2:12/36	3·6N 1·2:12/36	47.52	(4 df)	***
1·2N 3·6:12/36	3·6N 3·6:12/36	17.38	(4 df)	**
1·2N 1·2:12/67	6·7N 1·2:12/67	21.28	(4 df)	***
1·2N 6·7:12/67	6·7N 6·7:12/67	54.76	(4 df)	***
3·5N 3·5:35/36	3·6N 3·5:35/36	14.85	(4 df)	**
3·5N 3·6:35/36	3·6N 3·6:35/36	0.957	(4 df)	nsd
3·5N 3·5:35/67	6·7N 3·5:35/67	13.61	(4 df)	**
3·5N 6·7:35/67	6·7N 6·7:35/67	14.66	(4 df)	**
3·6N 3·6:36/67	6·7N 3·6:36/67	6.716	(4 df)	nsd
3·6N 6·7:36/67	6·7N 6·7:36/67	5.676	(4 df)	nsd
Comparisons of identical genotypes derived from different backgrounds				
1·2N 1·2:12/35	1·2N 3·5:12/35	180.7	(4 df)	***
1·2N 1·2:12/36	1·2N 3·6:12/36	47.52	(4 df)	***
1·2N 1·2:12/67	1·2N 6·7:12/67	129.4	(4 df)	***
3·5N 1·2:12/35	3·5N 3·5:12/35	20.34	(4 df)	***
3·5N 3·5:35/36	3·5N 3·6:35/36	10.30	(4 df)	*
3·5N 3·5:35/67	3·5N 6·7:35/67	10.92	(4 df)	*
3·6N 1·2:12/36	3·6N 3·6:12/36	11.19	(4 df)	*
3·6N 3·5:35/36	3·6N 3·6:35/36	2.014	(4 df)	nsd
3·6N 3·6:36/67	3·6N 6·7:36/67	10.23	(4 df)	*
6·7N 1·2:12/67	6·7N 6·7:12/67	14.80	(4 df)	**
6·7N 3·5:35/67	6·7N 6·7:35/67	11.36	(4 df)	*
6·7N 3·6:36/67	6·7N 6·7:36/67	19.38	(4 df)	***

P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different

A comparison of nuclear number distributions in conidia from derived homokaryons with their heterokaryotic parents (Table 4.11) reveals that in the majority of cases significant differences in patterns of production were observed. These differences were largely quantitative, and not necessarily qualitative. The least deviation from the heterokaryotic pattern was detected for those strains that were numerically less frequent in the heterokaryotic mycelium eg 1.2N 3.6mt: 1.2/3.6 from heterokaryon 1.2/3.6 3.6mt.

Patterns of germination of the derived conidia were similar in all cases. Regardless of the initial distribution, conidia with different numbers of nuclei were either equally germinable, or the multinucleate conidia germinated most rapidly. For the majority of strains this skewed the final distribution of nuclear numbers towards a prevalence of uninucleate conidia. For those strains that did not attain 100% germination within 48 h, the final distributions showed greater resemblance to the initial distribution patterns than at earlier sampling times (see Table 4.9a). This implies that a fixed proportion, or basal level of conidia were inviable, regardless of the actual numbers of nuclei that they possessed. Conidia from derived homokaryons attained both more rapid, and higher germination, than either the primary parental homokaryons, or the naturally-occurring homokaryons. Different nuclear genotypes derived from the same cytoplasmic background exhibited independent patterns of germination; in some cases, less disparity was observed when strains with identical nuclei but different cytoplasms were compared (Table 4.12).

In contrast to the derived homokaryons, naturally-occurring homokaryotic strains produced conidia that were mostly binucleate (Figure 4.8). In the majority of strains the distribution of nuclear numbers shifted towards a predominantly uninucleate status. For many strains this shift was not statistically significant, particularly when the overall germination levels were low (Table 4.9c).

Table 4.11. *Chi-squared, χ^2 (4 df), comparisons of nuclear number distributions in conidia from derived homokaryons with distributions in 'parental' heterokaryons.*

Comparisons of derived homokaryons with heterokaryotic parents				
Strain	XN Xmt	XN Ymt	YN Xmt	YN Ymt
1·2/3·5	210.9	12.103	43.43	111.2
1·2mt	***	*	***	***
1·2/3·5	207.5	6.122	43.52	108.4
3·5mt	***	nsd	***	***
1·2/3·6	22.71	1.779	12.53	15.20
1·2mt	***	nsd	*	**
1·2/3·6	10.66	7.482	27.56	31.02
3·6mt	*	nsd	***	***
1·2/6·7	90.08	34.72	25.82	10.14
1·2mt	***	***	***	*
1·2/6·7	80.99	43.62	19.31	3.516
6·7mt	***	***	***	nsd
3·5/3·6	141.9	75.28	76.07	72.78
3·5mt	***	***	***	***
3·5/3·6	151.59	85.69	87.23	84.55
3·6mt	***	***	***	***
3·5/6·7	61.31	34.63	73.49	24.54
3·5mt	***	***	***	***
3·5/6·7	39.35	18.36	56.40	15.07
6·7mt	***	**	***	**
3·6/6·7	51.54	28.80	102.6	36.28
3·6mt	***	***	***	***
3·6/6·7	14.94	26.54	40.62	7.479
6·7mt	**	***	***	nsd

P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different

Table 4.12. *Statistical examination of germination rates in derived homokaryons, comparing informative genotypes with different origins.*

Variability within genotypes (nuclei identical)				
1·2Ns	**3·5Ns**	**3·6Ns**		**6·7Ns**
χ^2=188.8 (6 df) ***	χ^2=18.47 (6 df) **	χ^2=404.9 (6df) ***		χ^2=254.7 (6 df) ***
Major contributors to variation				
1·2N 1·2:12/36	3·5N 3·5:35/36	3·6N 3·6:12/36		6·7N 67:35/67
1·2N 1·2:12/67	3·5N 3·6:35/36	3·6N 3·6:35/36		6·7N 67:35/67
Comparisons of different genotypes derived from the same background				
1·2N 1·2:12/35	3·5N 1·2:12/35	43.43	(1 df)	***
1·2N 3·5:12/35	3·5N 3·5:12/35	68.48	(1 df)	***
1·2N 1·2:12/36	3·6N 1·2:12/36	109.8	(1 df)	***
1·2N 3·6:12/36	3·6N 3·6:12/36	23.72	(1 df)	***
1·2N 1·2:12/67	6·7N 1·2:12/67	9.858	(1 df)	**
1·2N 6·7:12/67	6·7N 6·7:12/67	26.83	(1 df)	***
3·5N 3·5:35/36	3·6N 3·5:35/36	7.278	(1 df)	**
3·5N 3·6:35/36	3·6N 3·6:35/36	29.61	(1 df)	***
3·5N 3·5:35/67	6·7N 3·5:35/67	10.27	(1 df)	**
3·5N 6·7:35/67	6·7N 6·7:35/67	11.08	(1 df)	***
3·6N 3·6:36/67	6·7N 3·6:36/67	12.16	(1 df)	***
3·6N 6·7:36/67	6·7N 6·7:36/67	38.85	(1 df)	***
Comparisons of identical genotypes derived from different backgrounds				
1·2N 1·2:12/35	1·2N 3·5:12/35	188.0	(1 df)	***
1·2N 1·2:12/36	1·2N 3·6:12/36	25.15	(1 df)	***
1·2N 1·2:12/67	1·2N 6·7:12/67	35.73	(1 df)	***
3·5N 1·2:12/35	3·5N 3·5:12/35	1.335	(1 df)	nsd
3·5N 3·5:35/36	3·5N 3·6:35/36	15.81	(1 df)	***
3·5N 3·5:35/67	3·5N 6·7:35/67	0.141	(1 df)	nsd
3·6N 1·2:12/36	3·6N 3·6:12/36	101.8	(1 df)	***
3·6N 3·5:35/36	3·6N 3·6:35/36	2.726	(1 df)	nsd
3·6N 3·6:36/67	3·6N 6·7:36/67	3.367	(1 df)	*
6·7N 1·2:12/67	6·7N 6·7:12/67	0.974(1)	(1 df)	nsd
6·7N 3·5:35/67	6·7N 6·7:35/67	68.71(1)	(1 df)	***
6·7N 3·6:36/67	6·7N 6·7:36/67	1.363(1)	(1 df)	nsd

P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different

(1) Values for these genotypes were calculated at 12 h, all others were at 24 h.

Figure 4.7. Distribution of numbers of nuclei in ungerminated conidia from derived homokaryotic isolates of *H.annosum* at different times after plating out onto MEA at 20°C. Values shown are corrected for overall percentage germination (frequency at any given time x proportion of conidia not germinated). Sample sizes (number of conidia examined) for the 3,6,12,24 and 48 h sampling times were, respectively:(a) 1.2N 1.2mt :1.2/3.5 (444, 260, 86, 67); (b) 1.2N 1.2mt :1.2/3.6 (482, 543, 434, 538, 170); (c) 1.2N 1.2mt :1.2/6.7 (532, 545, 326, 197, -); (d) 1.2N 3.5mt :1.2/3.5 (543, 479, 257, 555, 363); (e) 1.2N 3.6mt :1.2/3.6 (521, 425, 214, 259, 170); (f) 1.2N 6.7mt :1.2/6.7 (511, 589, 524, 297, 150).

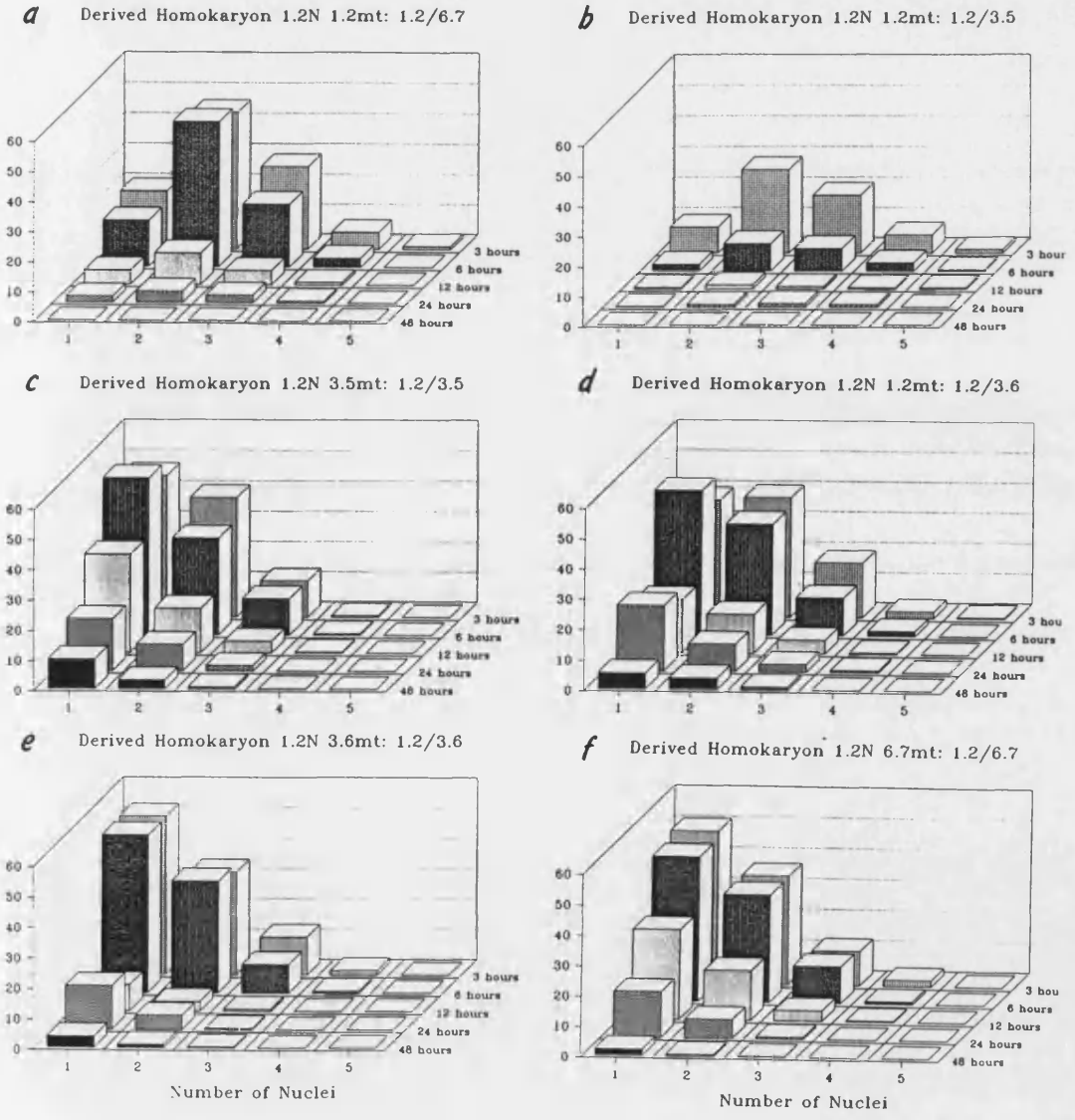


Figure 4.7 continued. Distribution of numbers of nuclei in ungerminated conidia from derived homokaryotic isolates of *H.annosum* at different times after plating out onto MEA at 20°C. Values shown are corrected for overall percentage germination (frequency at any given time x proportion of conidia not germinated). Sample sizes (number of conidia examined) for the 3,6,12,24 and 48 h sampling times were, respectively: (g) 3.5 N 3.5mt :1.2/3.5 (505, 413, 317, 234, -); (h) 3.5N 3.5mt :3.5/3.6 (524, 516, 417, 219, -); (i) 3.5N 3.5mt :3.5/6.7 (519, 443, 211, 230, -); (j) 3.5N 1.2mt :1.2/3.5 (518, 541, 414, 214, -); (k) 3.5N 3.6mt :3.5/3.6 (518, 539, 516, 234, -); (l) 3.5N 6.7mt :3.5/6.7 (660, 521, 223, 231, -).

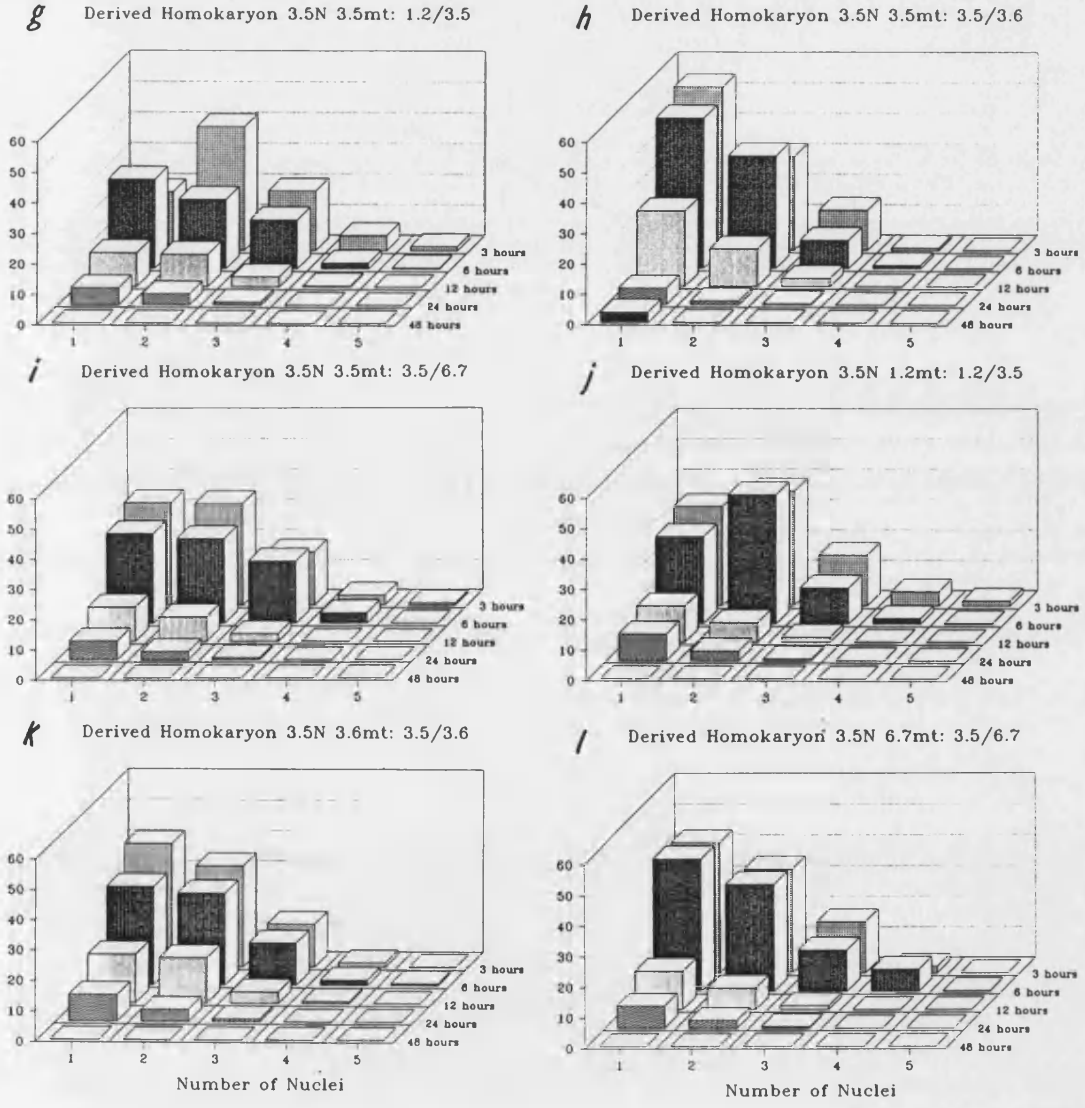


Figure 4.7 continued. Distribution of numbers of nuclei in ungerminated conidia from derived homokaryotic isolates of *H.annosum* at different times after plating out onto MEA at 20°C. Values shown are corrected for overall percentage germination (frequency at any given time x proportion of conidia not germinated). Sample sizes (number of conidia examined) for the 3,6,12,24 and 48 h sampling times were, respectively: (m) 3.6N 3.6mt :1.2/3.6 (534, 545, 219, 448); (n) 3.6N 3.6mt :3.5/3.6 (528, 505, 133, 149, -); (o) 3.6N 3.6mt :3.6/6.7 (365, 554, 215, 126, -); (p) 3.6N 1.2mt :1.2/3.6 (554, 529, 544, 169, -); (q) 3.6N 3.5mt :3.5/3.6 (527, 588, 235, -, -); (r) 3.6N 6.7mt :3.6/6.7 (537, 500, 213, 205, -).

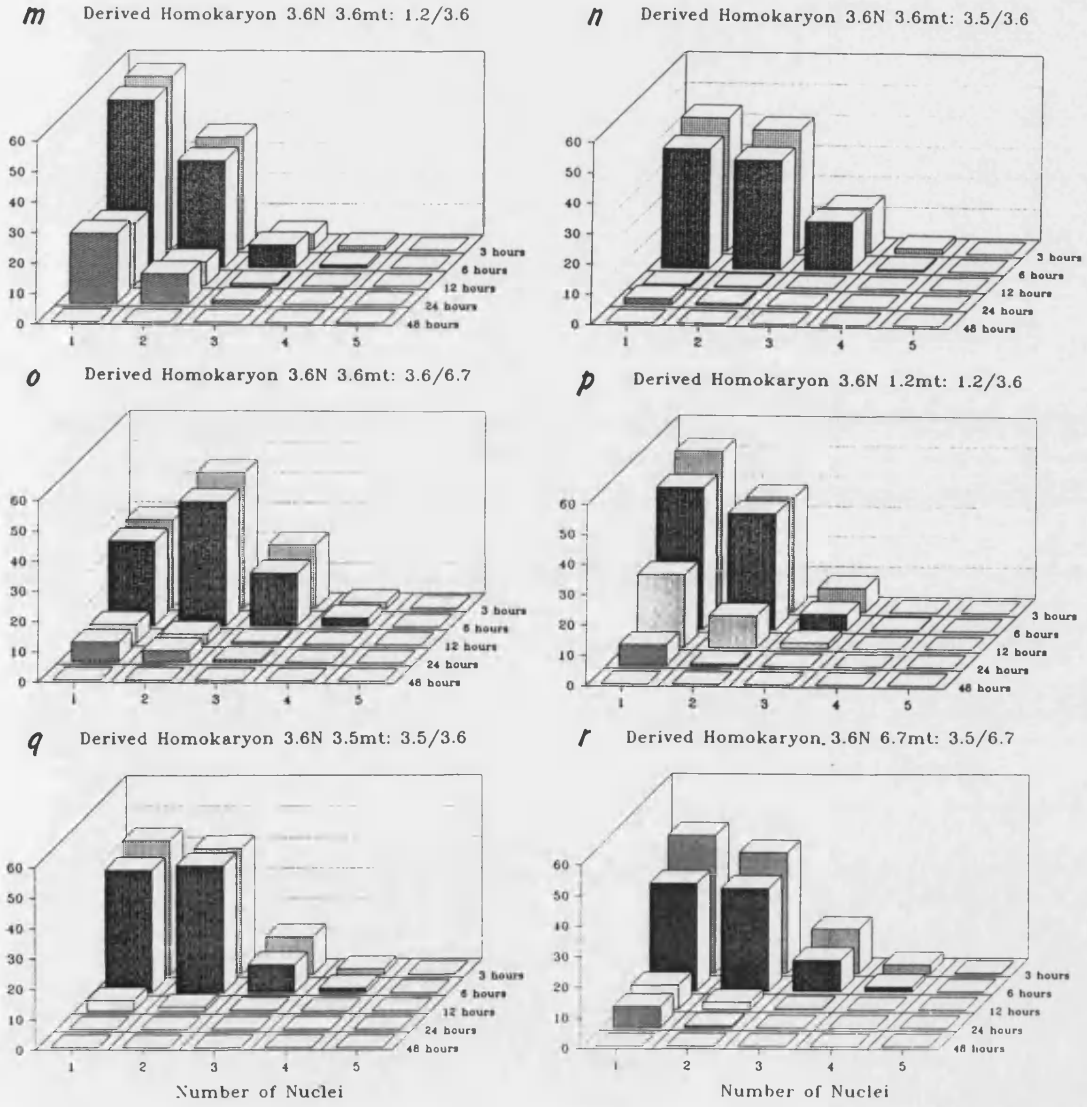


Figure 4.7 continued. Distribution of numbers of nuclei in ungerminated conidia from derived homokaryotic isolates of *H.annosum* at different times after plating out onto MEA at 20°C. Values shown are corrected for overall percentage germination (frequency at any given time x proportion of conidia not germinated). Sample sizes (number of conidia examined) for the 3,6,12,24 and 48 h sampling times were, respectively: (s) 6.7N 6.7mt :1.2/6.7 (522, 454, 183, -, -); (t) 6.7N 6.7mt :3.5/6.7 (504, 531, 282, -, -); (u) 6.7N 6.7mt :3.6/6.7 (470, 360, 113, -, -); (v) 6.7N 1.2mt :1.2/6.7 (508, 302, 112, -, -); (w) 6.7N 3.5mt :3.5/6.7 (519, 417, 134, -, -); (x) 6.7N 3.6mt :3.6/6.7 (503, 413, 38, -, -).

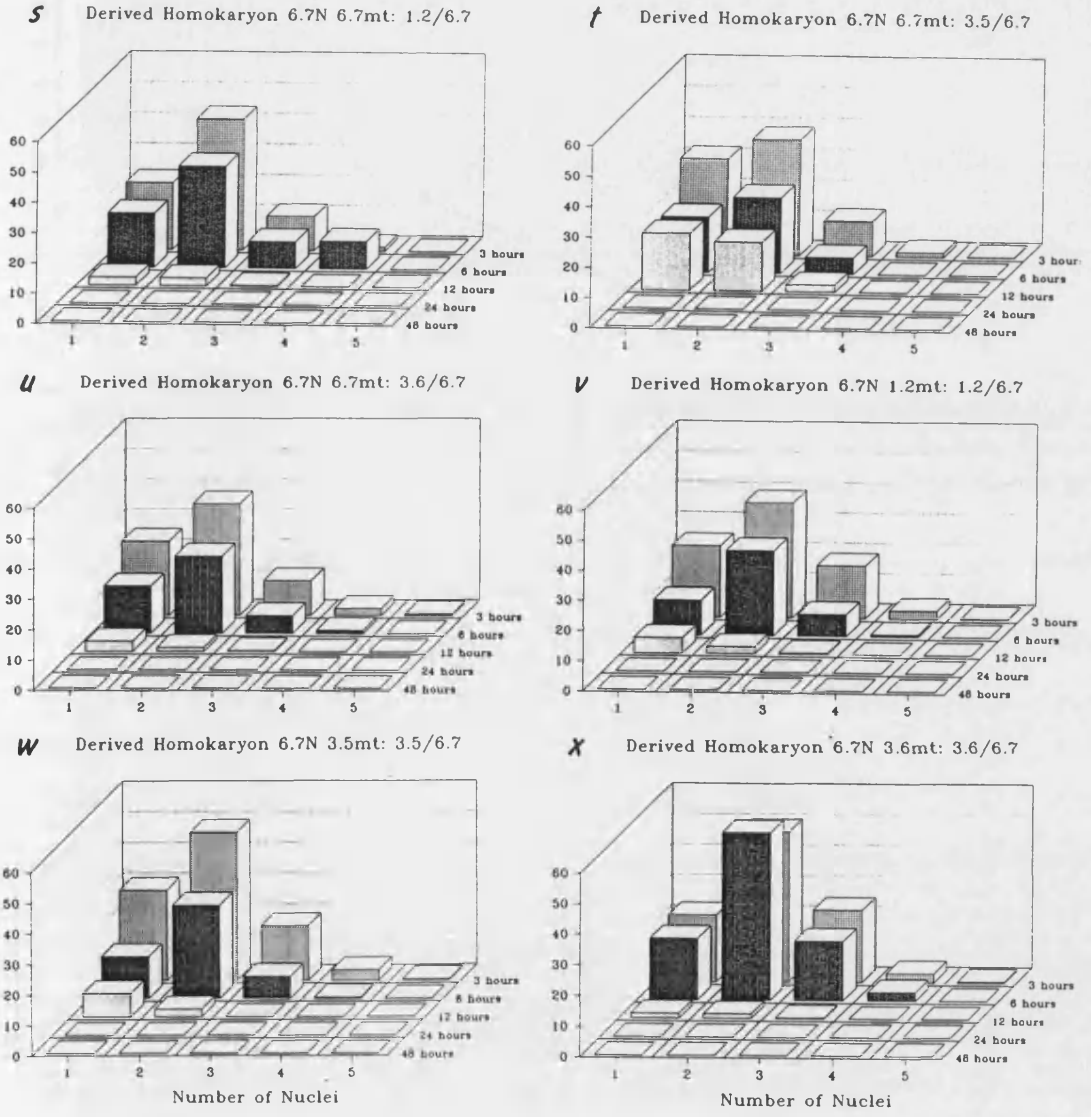


Figure 4.8. Distribution of numbers of nuclei in ungerminated conidia from naturally-occurring homokaryotic isolates of *H.annosum* at 3 and 24 h after plating out onto MEA at 20°C. Values shown are corrected or overall percentage germination (frequency at any given time x proportion of conidia not germinated). Sample sizes (number of conidia examined) for the 3 and 24 h sampling times were, respectively:(a) B2.1 (516, 385); (b) B2.2 (528, 427); (c) B2.3 (505, 282); (d) B2.4 (642, 137); (e) B2.5 (497, 315); (f) B2.6 (416, 336); (g) B2.7 (316, 273); (h) B2.8 (392, 313).

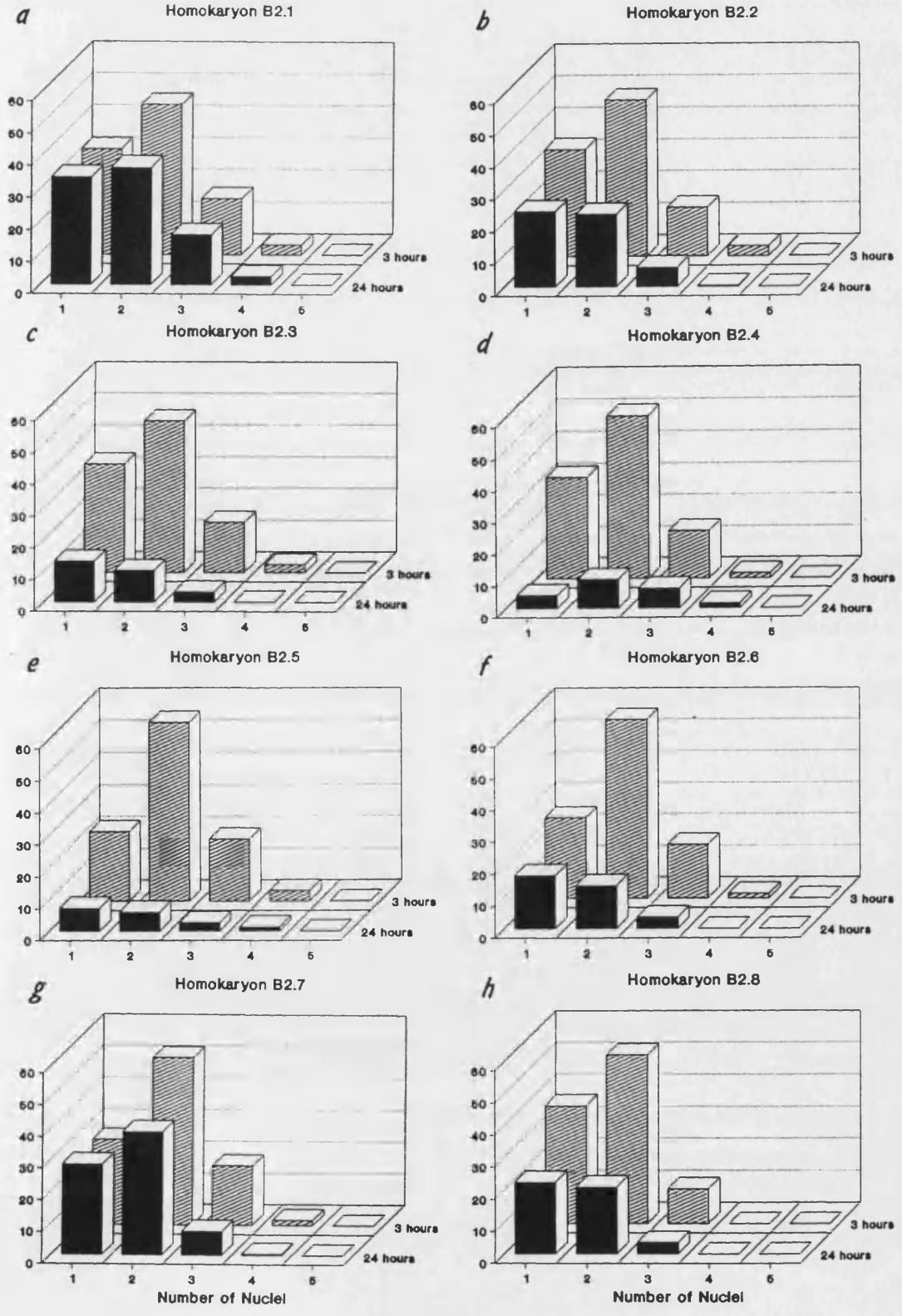
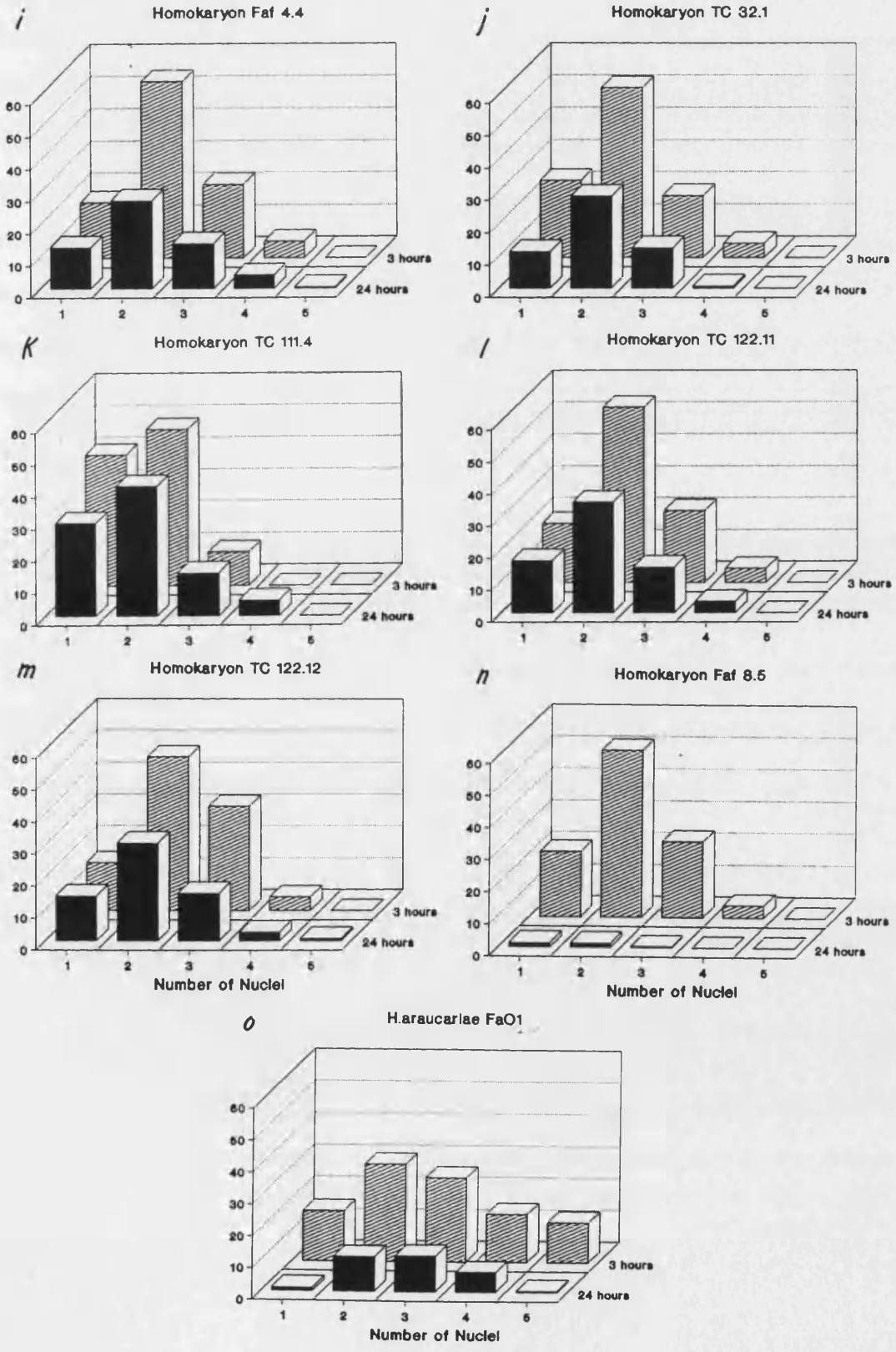


Figure 4.8 continued. Distribution of numbers of nuclei in ungerminated conidia from naturally-occurring homokaryotic isolates of *H.annosum* at 3 and 24 h after plating out onto MEA at 20°C. Values shown are corrected or overall percentage germination (frequency at any given time x proportion of conidia not germinated). Sample sizes (number of conidia examined) for the 3 and 24 h sampling times were, respectively: (i) Faf 4.4 (394, 448); (j) TC 32.1 (361, 370); (k) TC 111.4 (299, 215); (l) TC 122.11 (250, 239); (m) TC 122.12 (417, 229); (n) Faf 8.5 (423, 133) (o) FaO1 (201, 229).



DISCUSSION

The distribution patterns for the numbers of nuclei allocated to conidia for derived homokaryons were distinct from those shown by primary homokaryons. Whereas the progenitors of the experimental strains, and all of the natural homokaryons, produced conidia which were mostly binucleate (modal mean = 2), many of the derived homokaryons produced a higher proportion of uninucleate conidia (modal mean = 1).

When the nuclear numbers for derived conidia were examined closely it was difficult to establish any clear pattern that could account for their altered distributions. For those homokaryons that were numerically dominant in a heterokaryon (B3.5 > B3.6 > B1.2 > B6.7; see earlier results) the distributions were often not qualitatively different from those of their source heterokaryons. However, homokaryons lower in the dominance hierarchy were sometimes observed to revert to the production of binucleate conidia.

Homokaryons recovered from their resident mycelia generally produced conidia with higher mean numbers of nuclei than those recovered from the mycelium that they had invaded. Exceptions to this pattern were only observed when sib-related primary homokaryons were recovered from heterokaryons; here the homokaryons from resident mycelia often produced conidia with a slightly reduced mean. In these heterokaryons, the nuclear distributions actually did not differ greatly.

These observations suggest that the tendency to produce uninucleate conidia may become imprinted upon the nuclei during heterokaryosis, and that this property can then be maintained in a stable fashion as a result of a hidden epigenetic inheritance system. Moreover this tendency may be more strongly associated with invasive nuclei than those that remain within their resident mycelium.

Differences in the *identity* of the cytoplasmic partners for a given nuclear genotype surprisingly made no significant difference to either the rate of germination or to the pattern of germination. The rates of germination of the derived homokaryons and the final percentage germination after 48 h were higher than those shown by the primary homokaryons, perhaps as a result of the retention of other properties characteristic of heterokaryons such as those responsible for increased hyphal extension rates.

Of significance is that no conflict was apparent from the patterns of spore germination in any of the derived homokaryons when they were compared with their progenitor heterokaryons. This applied, regardless of whether or not the homokaryon retained a heterokaryon-like nuclear number allocation pattern, or reverted to a primary homokaryon pattern. In either case, the conidia were equally germinable, or the multinucleate conidia germinated more readily.

An important property of spore production in *Heterobasidion* becomes evident from an inspection of the analyses presented in Table 4.9a and b. The decrease in chi-square values for the 48h comparison reveals that the distribution was returning to its initial range. Although germination approached 100% after 48 h in most cases, the few remaining ungerminated spores had a nuclear number distribution similar to that at the start of the experiment. This implies that a small, but fixed, proportion of conidia were inviable regardless of their nuclear allocation or past associations.

Some derived homokaryons could not be distinguished from their progenitor homokaryons, but most displayed some alterations in morphology and behaviour as a result of their past association with different non-self partners. Moreover, most of these changes involved the retention of properties more often associated with heterokaryons. The continued production of pseudosclerotial plates, watery droplets and more restricted production of conidiophores are all examples of such behaviour.

The origins of these '*acquired characteristics*' are not clear from this study alone, though it is possible to speculate that if heterokaryosis necessitates a switch from one set of genetic programs to another (Raper, 1966) the removal of nuclei from a heterokaryon may not lead to a complete loss of the differentiated state. If the heterokaryotic state is passively maintained then there might not be any further requirement for the sustained input of controlling factors (eg nuclei of complementary mating-type).

Alternatively, the stability of the characteristics associated with heterokaryosis shown by the derived homokaryons could be the result of *hyperepigenetic* inheritance systems. These result from the feedback of internally driven non-genetic processes that generate a self-sustaining organizational state (Rayner, Ramsdale & Watkins, 1995). These are analogous to the steady-state or self-perpetuating metabolic patterns recently reviewed by Jablonka & Lamb (1995).

Strohman (1994) also proposed that any regulatory network could establish a persistent functional 'metastable' state through positive feedback; even in the absence of sustained inputs from inducers - see Blau (1992) for other examples in eukaryotes.

The direct transmission of the trait for pseudosclerotial plate production from one strain to another is a good candidate for a metastable state determined by just such a hyperepigenetic inheritance system. The production of PSPs and many other emergent structures in fungi is often accompanied by the production of a range of polyphenolic compounds which are capable of interacting with, and generating free-radicals and reactive oxygen species. Once these compounds have been elicited, their production may be maintained purely as a result of chemical autocatalysis, or as a result of positive feedback on transcriptional regulators.

The patterns of inheritance of PSP production could also be attributed to the transfer of genetic information from one nucleus (B3.6) to another, ie a form of transformation. Alternatively, the regulatory pathways required for PSP production could be initiated by the B3.6 nuclei and then maintained as a result of the stable maintenance of epigenetic chromosomal marks.

Imprinting in fungi has not been widely recorded, but a number of examples within the literature imply its occurrence. The continued production by monokaryons of characters normally associated with dikaryons has been reported following de-dikaryotization in *Hirschioporus abietinus* (Harder, 1927b), *Coprinus disseminatus* (Butler, 1972), *Flammulina velutipes* (Aschan, 1952), *Polystictus* (*Coriolus*) *versicolor* (Lange, 1966) and *Schizophyllum commune* (Parag, 1970; Raper & Raper, 1964; Raper & San Antonio, 1954; Wessels, Hoeksema & Stemerding, 1976; Raper, 1985). In these examples, clamp-cells and pseudoclamps were observed on monokaryotic neohaplonts for many cell division cycles after their removal from dikaryons. Neohaplonts from *Hirschioporus* (Fries & Aschan, 1952) and *Trichoderma* (Stasz & Harman, 1990) were morphologically and physiologically more variable than neohaplonts of the same genotype derived from homokaryons. Using isoenzyme markers, Stasz & Harman (1990) could not distinguish between neohaplonts recovered from heterokaryons and their source homokaryotic strains. They inferred from this that no parasexual events had occurred - however such a conclusion must be viewed with some caution

since isoenzymes are poor indicators of recombination. A sustained capacity for differentiation in the absence of genetic inputs has also been reported in other eukaryotes, including *Acetabularia* (Goodwin, 1995).

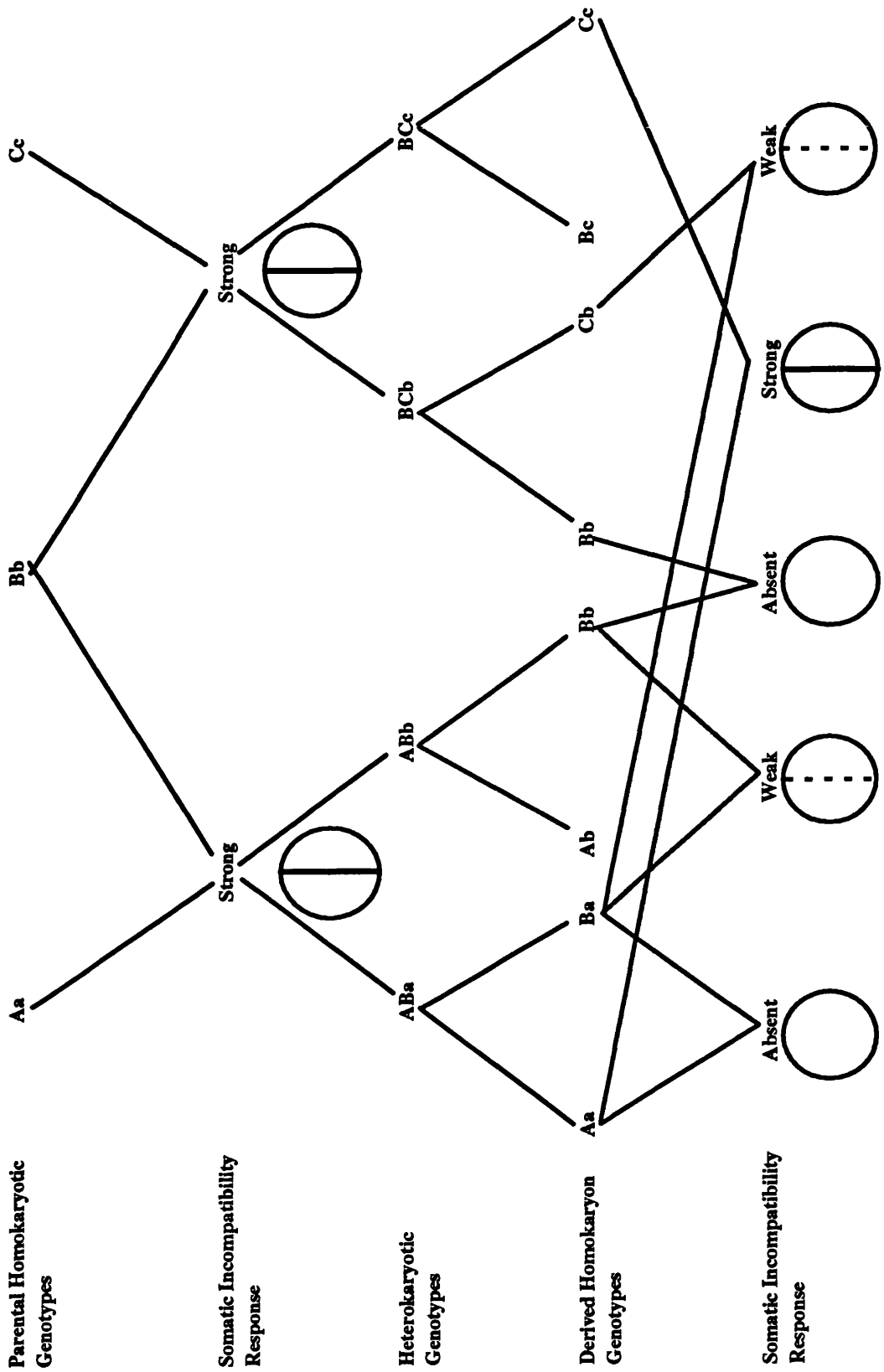
Strains B3.5 and B3.6 were virtually homo-allelic for their nuclear RFLP markers which may account for the similarity in their behaviour. Several studies have examined variation within populations of naturally-occurring heterokaryons of *Heterobasidion annosum* using DNA fingerprinting probes (Karlsson, 1993; Kasuga & Mitchelson, 1993; Kasuga *et al.*, 1993; Fabritius & Karjalainen, 1993). However, none of these studies have critically examined the inheritance of these markers, or looked at the variation between the partners in a heterokaryon.

Differences in mitochondrial genotype had no tangible effect upon the behaviour of either homokaryons or heterokaryons. With only one exception, homokaryons with identical nuclei but different associated mitochondrial partners demonstrated no significant differences in overall growth form. Furthermore, as already described, no effect was observed upon metrical traits such as nuclear number distribution, rates of germination or overall germination levels. The neutrality of the cytoplasm with respect to phenotypic expression could be attributed to the relative genetic homogeneity of the strains examined. Despite some differences in their mitochondrial DNA haplotypes, the source of the variation and its relevance to the activity of the mitochondria themselves was not established. Since both mitochondrial genotypes generated the same hybridization pattern when used as probes against each other, they were probably very similar. Consequently these strains may not have been the most appropriate to examine the effects of cytoplasmic variation upon phenotypic variation.

Some of the most significant changes shown by the derived homokaryons concerning their somato-sexual responses are summarized in Figure 4.9. One of the many surprises in this study was that differences in the cytoplasmic background of homokaryons with identical nuclei could result in somatic incompatibility.

Most of the alterations in behaviour can be interpreted in terms of the retention of the epigenetic blueprint established during the stabilization of a harmonious relationship between two mating-type compatible nuclei. Consequently when two nuclei are separated from a heterokaryon and are then re-associated, they do not express any of the incompatibilities that

Figure 4.9. Summary of interaction outcomes between primary parental and secondary derived homokaryons of *Heterobasidion annosum*, emphasizing nuclear genotype, mitochondrial genotype and 'past-history' or association.



they expressed when they first met. This scenario supports an earlier suggestion made by Rayner *et al* (1984) that mating-type compatible responses involve the *override* of somatic incompatibility.

An appreciation of the potential for the acclimation of nuclei in an association has important ramifications for our understanding of both the developmental biology and population biology of basidiomycetous fungi. Acclimation ensures that nuclei will associate more readily with partners that they have already met than with ones that they haven't. This could enhance the fitness of an individual by increasing the frequency with which it entered into harmonious relationships relative to deleterious relationships. Furthermore, should a heterokaryotic mycelium breakdown into its components (through drift or selection), the presence of a cellular memory system might prevent the subdivision of the heterokaryon into discrete unstable domains (cf Ainsworth *et al.*, 1992).

SECTION V

PROTOPLAST RECOVERY OF HOMOKARYOTIC GENOTYPES FROM *STEREUM HIRSUTUM* (WILLD. EX FR.) / *STEREUM COMPLICATUM* (FR.) HETEROKARYONS: RATIO ASYMMETRY AND POST-GERMINATION MORTALITY

INTRODUCTION

Work with *Heterobasidion annosum* has indicated that instabilities arising from allopatric matings may be of fundamental importance to understanding the influence of genomic conflict on speciation processes and in particular, the ability of heterokaryons with highly disparate genomes to remain viable. An opportunity for examining this issue arises from work that has been done on allopatric matings amongst members of the genus *Stereum.*

Stereum hirsutum is a widespread wood-decay basidiomycete that produces primary and secondary mycelia bearing whorled (verticillate) clamp-connections. Extensive investigations have revealed that both out-crossing and non-outcrossing populations occur (eg Coates *et al.*, 1981; Boddy & Rayner, 1982; Rayner & Turton, 1982; Rayner *et al.*, 1984; Ainsworth, 1986,1987; Ainsworth & Rayner, 1989). Mating is controlled by a multi-allelic unifactorial homogenic incompatibility locus, or C-factor (Coates, Rayner & Todd, 1981). Complementarity of mating-type allows the bilateral formation of secondary mycelia.

Somatic incompatibility responses occur between heterokaryons and some combinations of homokaryon, which led Rayner *et al* (1984) to propose that mating-type complementarity can override somatic incompatibility in fully compatible pairings.

Heterozygosity at a second, unlinked multi-allelic locus (B-factor), causes a distinctive 'bow-tie' interaction in pairings between mating-type (C-factor) incompatible mycelia (Coates & Rayner, 1985a). In such pairings, the nuclei of one partner partially or completely replace the nuclei of the other. A rapid expression of somatic incompatibility limits the extent of this invasion, whereas a slow response can lead to complete take-over.

Allopatric matings of *Stereum* species have been shown to exhibit complex patterns of nuclear exchange, associated with varying degrees of cellular degeneration and emergence of variable heterokaryotic phenotypes (Ainsworth & Rayner, 1989; Ainsworth *et al.*, 1992). In one particular case, pairings between some strains of *S.hirsutum* and *S.complicatum* generated heterokaryons with a uniform appearance on the *'complicatum'* side, and heterokaryons with a heterogeneous appearance on the *'hirsutum'* side (Ainsworth *et al.*, 1992).

Heterokaryons recovered via single hyphal-tips from different regions of interactions between *S.complicatum* and *S.hirsutum* displayed a range of phenotypes that were distinct from, but

similar to, one or other of their parental strains. All heterokaryons recovered from the *complicatum* side of pairings were morphologically similar to the *complicatum* parent (pure white, flattened, felty colonies) but they possessed some altered somato-sexual recognition responses and were also capable of exhibiting light-induced phenotypic switches. Heterokaryons from the *hirsutum* side of interactions were either *complicatum*-like, or produced mycelia with a variety of forms that were similar to the *hirsutum* parent (sienna-ochraceous, dense aerial fluffy to fascicular colonies). Colonies with a *complicatum*-like appearance were referred to as C-type heterokaryons and the remainder as H-type heterokaryons (Ainsworth *et al.*, 1992).

Molecular analysis of the different classes of heterokaryon revealed that in some heterokaryons, markers specific to portions of the *hirsutum* genome were missing. Furthermore this partially correlated with the level of penetrance of the *hirsutum* phenotype; those strains with the full *hirsutum* complement were *hirsutum*-like, and those lacking the full *hirsutum* genome were *complicatum*-like. Not surprisingly, the genetically deficient heterokaryons exhibited altered somato-sexual recognition responses compared with those heterokaryons with all of the parental markers.

The patterns of genetic exchange and heterokaryon emergence that were observed in *Stereum* have been attributed to a combination of both genetic and epigenetic phenomena (Ainsworth *et al.*, 1992; Rayner, Ramsdale & Watkins, 1995), not least the establishment of different nucleo-cytoplasmic relationships on either side of interaction zones.

This section examines further the complex relationship between genetic content and the manifestation of phenotype in this developmentally indeterminate organism. Since the notion of 'a cell' within the holocoenocytic basidiomycetes extends to the entire domain occupied by a mycelium, some of the issues raised by genomic conflict can be examined in terms of the dynamics of the organelles populating them. In particular the question of how genetic information can be partitioned in space and time, and how the interactions between nuclei and mitochondria can have stable or unstable outcomes are addressed.

MATERIALS AND METHODS

Strains, subculturing and pairings

Unless otherwise stated, all strains were grown at 20°C in darkness on 90 mm diameter unvented Petri dish plates containing approximately 15 ml MEA. Stock-cultures of all strains were maintained at 4°C on MEA bijou slopes under sterile mineral oil.

The use of the homokaryotic progenitor strains EP18.7 (*S.hirsutum*) and JG1.5 (*S.complicatum*) to generate single hyphal-tip derived heterokaryons has been described in detail elsewhere (Ainsworth & Rayner, 1989; Ainsworth *et al.*, 1992). Table 5.1 lists the genotypic designations and phenotypic characteristics of the strains examined in this study. The single hyphal-tip derived heterokaryons examined were representative of the range of stable final colony types generated in the original investigations.

EP18.7 produced dense fluffy to fascicular sienna-ochraceous mycelial mats which contrasted with the flattened felty, pure white colonies of JG1.5. These characteristics, in combination with pairing experiments ensured that the two strains could be reliably distinguished following their recovery from heterokaryons.

Protoplast production

Initially a variety of protoplast-generating agents were utilized, both alone and in combination. These included chitinase, N-acetylglucosaminidase, Trichoderma lysing enzyme and Novozyme 234. In addition, three osmotica were examined, 1M $MgCl_2$; 1M sucrose and 0.5M $CaCl_2$ in 0.005M McIlvaines citric acid buffer (pH4.6). Neither chitinase nor N-acetylglucosaminidase resulted in any disruption of test *Stereum* cultures using the different buffers. Trichoderma lysing enzyme (SIGMA, UK) and Novozyme 234 produced considerable cellular disruption but no intact protoplasts with 1M $MgCl_2$ or 1M sucrose. Both the Trichoderma lysing enzyme and Novozyme 234 in combination with 0.5M $CaCl_2$ in 0.005M McIlvaines citric acid buffer (pH4.6) led to the production of large quantities of stable protoplasts.

The length of time for protoplasts to emerge was affected by the particular batch of enzymes used and their concentration, the age of the protoplasting solution and the age of the sample mycelium. For example Trichoderma lysing enzyme solution at a concentration of 0.7 mg ml^{-1},

Table 5.1. *Strain designations, nucleo-cytoplasmic status and phenotypes of 'hirsutum-complicatum' heterokaryons*

Strain	Nuclei*	Cytoplasm*	Phenotype	Protoplasts	Regenerants
EP18·7	EP	EP	H	Y	Y
JG1·5	JG	JG	C	Y	Y
7·69	EP/JG	EP	H	Y	Y
7·73	EP/JG	JG	C	Y	Y
7·77	EP/JG	JG	C	Y	N
8·78	EP/JG	JG	C	Y	Y
9·81	EP/JG	JG	C	Y	Y
9·83	EP/JG	JG	C	Y	Y
9·85	EP/JG	EP	H	Y	Y
9·87	EP/JG	JG	C	Y	Y
9·89	EP/JG	EP	C	Y	Y
9·91	EP/JG	JG	C	Y	N
9·95	EP/JG	EP	H	Y	Y
9·97	JG	JG	C	Y	Y
9·99	EP/JG	JG	C	Y	Y
10·82	EP/JG	JG	C	Y	Y
10·84	EP/JG	JG	C	Y	N
10·86	EP/JG	JG	C	Y	Y
10·88	EP/JG	JG	C	Y	N
10·90	EP/JG	JG	C	Y	N
10·92	EP/JG	JG	C	Y	Y
10·96	EP/JG	JG	C	Y	N
10·98	EP/JG	JG	C	Y	N
10·100	EP/JG	EP	H	Y	Y

* Genotypes were confirmed by Ainsworth *et al* (1992) using nuclear DNA single, dual and minisatellite fingerprinting techniques and by mitochondrial DNA haplotyping.

The progenitor strains used in this study were donated to Martyn Ainsworth by J Ginns (JG1.5) and E Parmasto (EP18.7). JG1.5 was a single basidiospore isolate obtained from a fruitbody growing on *Fagus* in New York, USA. EP18.7 was isolated as a single basidiospore from a fruit body found growing on *Populus* in the Ukraine, CIS.

first produced protoplasts after 20 min at 20°C but after only 3-4 min at 7 mg ml^{-1}. Protoplast solutions kept overnight at 4°C showed a ten-fold increase in the time to initial protoplast emergence. Young mycelia (4-d-old), protoplasted more rapidly than 7-d-old mycelia, which in turn protoplasted more rapidly than 30-d-old mycelia. Both homokaryotic and heterokaryotic mycelia were readily protoplasted using these enzymes. Protoplasts remained stable in fresh osmoticum for up to 7-d at 4°C, but some loss of viability could be detected after this time.

For the large scale production of protoplasts (approximately 1 x 10^9), mycelial plugs, 6 mm in diameter, were taken from the edge of actively growing 7-d-old cultures and were used to inoculate 250 ml yeast peptone broth (YPB: 10 g l^{-1} Lab M yeast extract powder, 10 g l^{-1} Lab M mycological peptone; sterilised at 110°C for 10 min). The resulting cultures were incubated at 20°C and shaken vigorously to break up mycelial mats once per day. After 7-d, mycelia were harvested, washed once with sterile distilled water, and once with protoplasting buffer (PB: 0.005 M McIlvaines sodium citrate / citric acid buffer pH 4.6, 0.5 M $CaCl_2$). The washed mycelia were then transferred to sterile tubes containing 25 ml protoplasting solution (7 mg ml^{-1} Sigma Trichoderma lysing enzyme in PB) and incubated at 20°C for 1 to 24 h. Subsamples of the mixture were monitored at regular intervals to determine when protoplast formation was complete.

Mycelial debris was removed from the mixture by passing it through two layers of sterile muslin, and then the protoplast suspension was centrifuged at 2600 g for 5 min. The supernatant was removed and the protoplast pellets were washed several times in 1.0 ml PB and then resuspended in 0.5 ml PB.

Small scale protoplast production, suitable for the direct analysis of genotype recovery rates was achieved by adding 1 ml protoplasting solution to single 6 mm diameter plugs cut directly from the interaction plates. Widespread application of this technique for examining interaction outcomes was not feasible since samples from old (28-d) mycelia produced protoplasts far less readily than from younger mycelia (<14-d-old) and furthermore they were less germinable.

Protoplast regeneration

Diluted protoplast suspensions (10^4 ml^{-1}) were used to inoculate an equivalent volume of protoplast regeneration broth (PRB: 25 g l^{-1} Lab M nutrient broth No. 2, made with PB). The protoplasts were incubated in stationary culture at 20°C for 24 - 48 h. Regeneration was monitored at regular intervals during this period. When significant protoplast regeneration was observed (>90%), samples were diluted in series with PB, and then spread directly onto MEA plates. After 24 h growth at 20°C, single germlings were picked off the plates after marking their position with a sterile dummy objective fitted with a 1 mm diameter cutting tube. Single protoplast cultures were transferred to Repli dishes containing 3 ml MEA. The dishes were examined after 7-d, and again after 28-d, when the identity of the isolates was more readily ascertained. Transient exposure to light after 7-d greatly aided the assignment of strain identities as a result of the 'light-induced' phenotypic switch shown by C-type heterokaryons (Ainsworth *et al.*, 1992).

The frequency of recovery of different phenotypic classes was recorded, along with the number of protoplast germlings that failed to develop after transfer to Repli dishes (an indication of the level of post-germination mortality or PGM).

Nuclear numbers in protoplasts

Protoplast suspensions were spread onto glass microscope slides and allowed to air-dry. Preparations were then flooded with aqueous (DAPI) and then examined at x400 magnification using an Olympus BH-2 microscope equipped with a BH-RFL-W epifluorescence unit. Illumination was provided using a 20 UV-W (UG1) excitation filter, BH-DMUV (DM400 + L420) dichroic mirror combination and an L435 barrier filter. The numbers of nuclei in at least 200 protoplasts were counted for each of the strains examined.

Confirmatory pairings

Fifteen representative protoplast-derived phenotypes from each of the strains successfully regenerated in this investigation were paired against each other, and against tester progenitor strains (homokaryotic parents and single hyphal-tip heterokaryons). Patterns of interaction and

mycelial emergence were followed, with particular attention being paid to the intensity of somatic incompatibility reactions and rates of nuclear migration.

One of the strains, an H-type heterokaryotic derivative of strain 10.86, and some of its protoplast-derived progeny, were studied in more detail. As in the confirmatory pairings, this strain was paired with each of the parental homokaryons and its progenitor heterokaryon; subsamples were then taken from the interaction plates to generate further protoplast cultures. The frequency with which the different phenotypes were recovered from these sub-samples were then recorded.

Statistical analyses

Data for nuclear number distributions in protoplasts were analyzed using standard χ^2 tests (Milton & Tsokos, 1983). Linear regression analysis was used in conjunction with Pearson's correlation coefficient to examine the relationship between the recovery of phenotypes and the level of post-germination mortality.

RESULTS

Colony characteristics and interaction outcomes

Experiments repeating the initial pairings of Ainsworth *et al*, 1992, between *S.hirsutum* (EP18.7) and *S.complicatum* (JG1.5) produced qualitatively similar results. Interactions between the homokaryons resulted in the formation of morphologically uniform heterokaryotic mycelia on the *complicatum* side of the interaction interface, and heterogeneous heterokaryotic mycelia on the *hirsutum* side (Figure 5.1). *Complicatum* nuclei migrating into *hirsutum* mycelia induced a flattening of the *hirsutum* homokaryon resulting in the production of degenerate sienna-olivaceous mycelia. Migration fronts were demarcated by watery droplets along the interface between the homokaryotic and degenerative mycelium. Gradually islands of *hirsutum*-like and *complicatum*-like mycelium emerged from the degenerate zone; first at the edge of the colonies and then progressively closer to the inoculum plugs.

Regeneration of protoplasts

After 24-48 h incubation in nutrient broth containing 0.5M $CaCl_2$ and 0.005M McIlvaines citrate buffer, protoplasts from most strains regenerated to produce germ-tubes (Table 5.1). Those that failed to regenerate were all derived from C-type heterokaryons and were mostly strains generated in week 5 of the original experiments of Ainsworth *et al*, 1992.

Several regenerating protoplasts appeared to 'bud', producing small daughter cells which remained attached to the parental protoplast 'cell'. Sometimes this process was accompanied by the transfer of the entire cytoplasm from parent to daughter cell, leaving behind a protoplast ghost. Such behaviour indicates that a wall had been synthesized around the regenerating protoplast. Chains of 'budding' cells were frequently observed, with any one parent cell only giving rise to a single daughter. Single germ-tubes then emerged from the last and smallest daughter cells (Figure 5.2).

Recovery of genotypes

Four distinct colony types could be recovered from protoplasted heterokaryotic mycelia. Colonies that both resembled and behaved in pairings like the parental strains were classified as homokaryons. Strains that resembled the *complicatum* parent, and produced a light-induced phenotypic switch around the inoculation point following brief exposure of the

Figure 5.1. (a) Parental '*complicatum*' homokaryon, JG1.5; (b) parental '*hirsutum*' homokaryon, EP18.7; (c) original interaction plate between EP18.7 - right, and JG1.5 - left (d-f) a selection of heterokaryotic hyphal-tip progeny taken from the interaction plates.

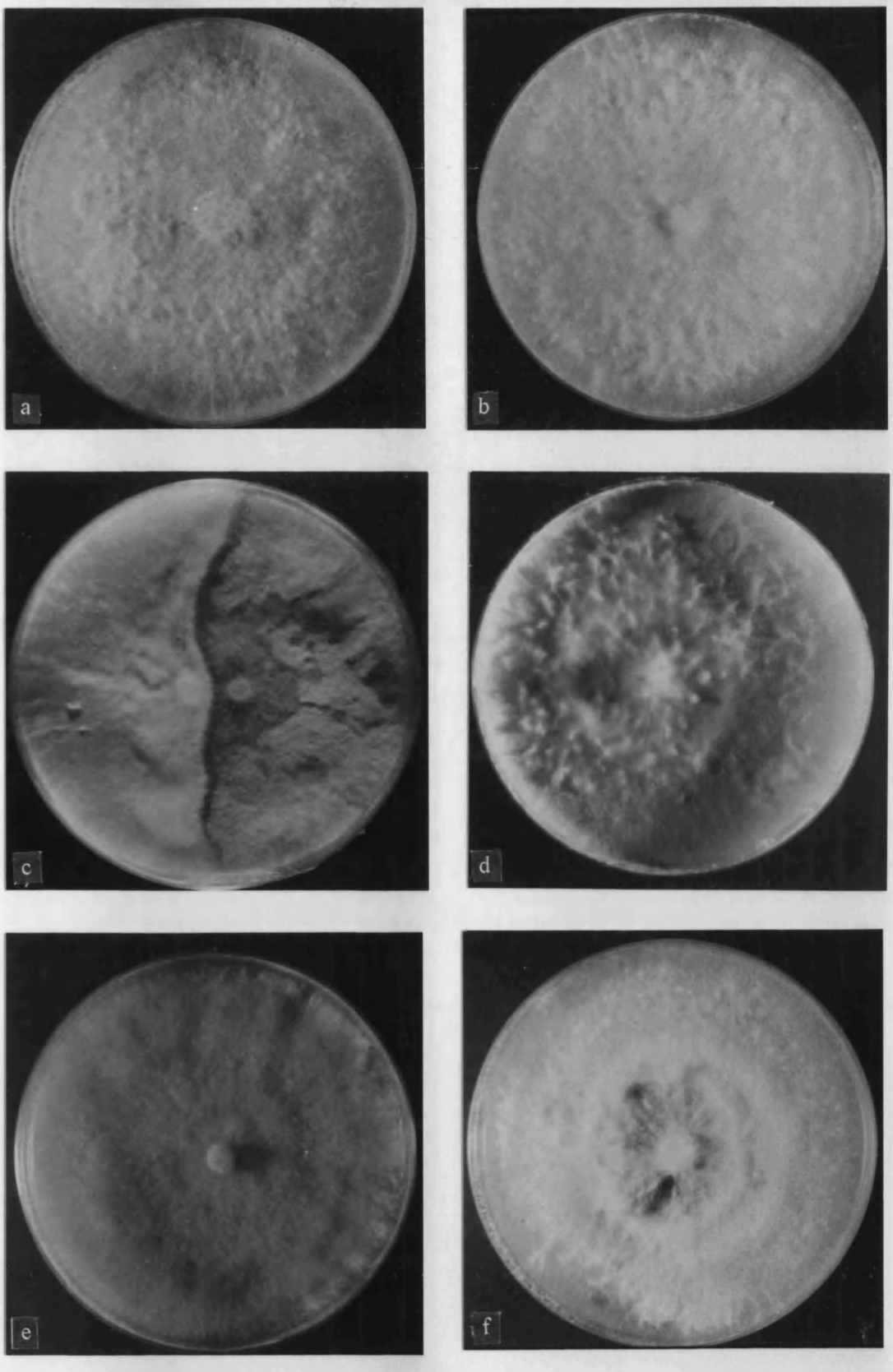
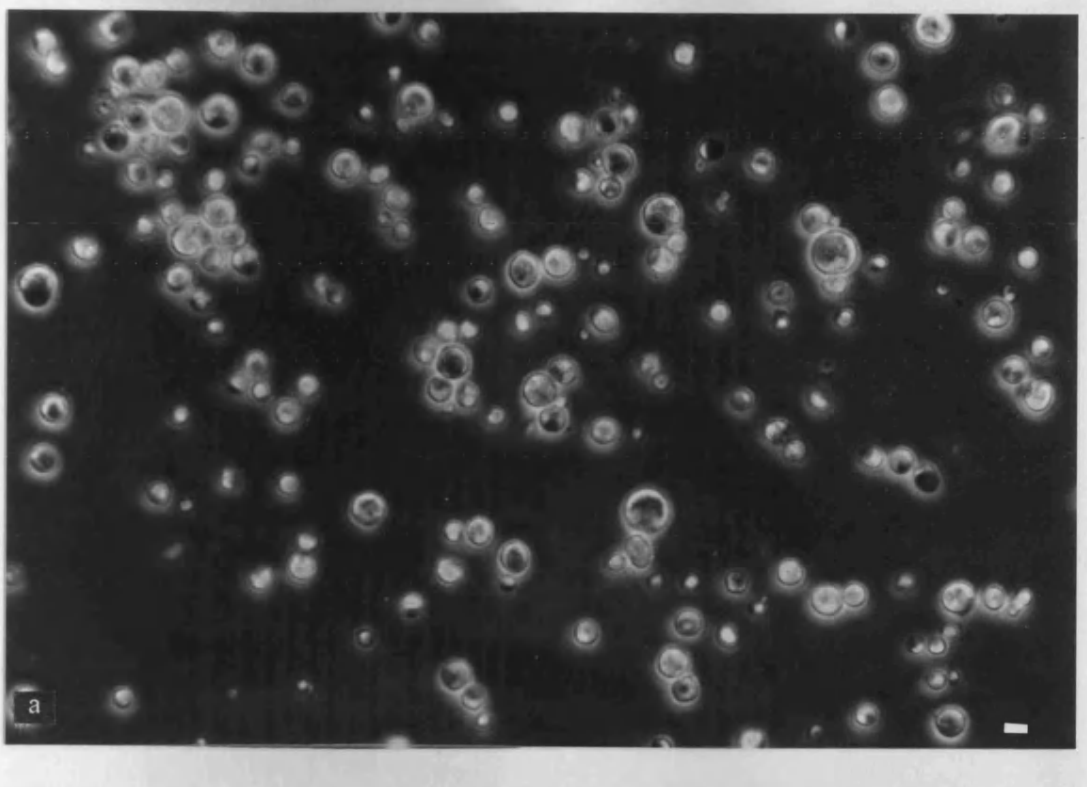


Figure 5.2. Phase-contrast micrographs shoving: (a) newly formed protoplasts derived from '*hirsutum-complicatum*' heterokaryon, 10.86and; (b) regenerating protoplasts, showing sequential 'budding' and terminal cell size reduction followed by germ-tube emergence. Scale bars equal 5 μm.



plates to light, were classified as C-type heterokaryons. More variable colony types that produced luteous-sienna appressed mycelia and exhibited somatic incompatibility responses against parental *hirsutum* strains were classified as H-type heterokaryons.

The frequency with which the different types of colony were recovered varied between strains (Table 5.2). In many cases fewer homokaryotic genotypes were recovered than was expected from a consideration of the distribution of nuclear numbers in protoplasts.

Although protoplasts could contain up to seven nuclei (Figure 5.3), most were uninucleate (30% - 48%) and should therefore have germinated to produce a high proportion of homokaryons. A chi-squared (84 df), comparison of the allocation of numbers of nuclei to protoplasts for all of the heterokaryotic strains examined revealed that there were some differences between genotypes; χ^2(84 df) = 141.20, P<0.001. However, when this χ^2-value was partitioned, two of the strains examined contributed over 36% of the total variation. Re-calculation of the statistic without these two strains gave no indication of significant differences between genotypes; χ^2(76 df) = 89.93, P<0.100. The patterns of allocation of nuclei to protoplasts deviated slightly from a truncated Poisson distribution corrected for the absence of zero-values (χ^2 (4 df) = 10.45, P < 0.05).

Some of the H-type heterokaryons examined produced mostly *hirsutum* homokaryons and H-type heterokaryons. However H-type heterokaryons, 7.69 and 10.90, both produced large numbers of *complicatum*-like colonies. Overall very few H-type heterokaryons were recovered. In general, as the numbers of *complicatum* homokaryons recovered from heterokaryons increased, the number of hirsutum heterokaryons recovered decreased.

With regard to the suspected underlying nuclear ratios, some heterokaryons of both the C-type and H-type were almost entirely made up of a single genotype.

Post-germination mortality

A direct relationship was found between the level of post-germination mortality observed for a given strain and the recovery of homokaryons (Figure 5.4,5.5). Pearsons' product moment correlation coefficient (r_p) for C-type heterokaryon data indicated a highly significant linear relationship (r_p = -0.837, P<0.001). As the proportion of homokaryons recovered from C-type heterokaryons decreased, the levels of post-germination mortality increased. For H-type

Figure 5.3. Distribution of numbers of nuclei in protoplasts derived from '*hirsutum-complicatum*' heterokaryons. Strains are presented in rank order from the highest mean number per cell, strain 9.91 (2.4 per cell) to the lowest, strain 10.88 (1.72 per cell). Sample sizes (number of protoplasts examined) were, respectively: 9.91 (137); 10.98 (157); 9.99 (208); 9.87 (369); 10.86 (207); 9.97 (216); 9.89 (189); 10.96 (207); 9.81 (210); 10.100 (212); 10.84 (170); 10.82 (222); 9.95 (214); 7.69 (215); 10.92 (221); 7.77 (210); 7.73 (211); 10.90 (130); 6.54 (379); 8.78 (209); 10.88 (247).

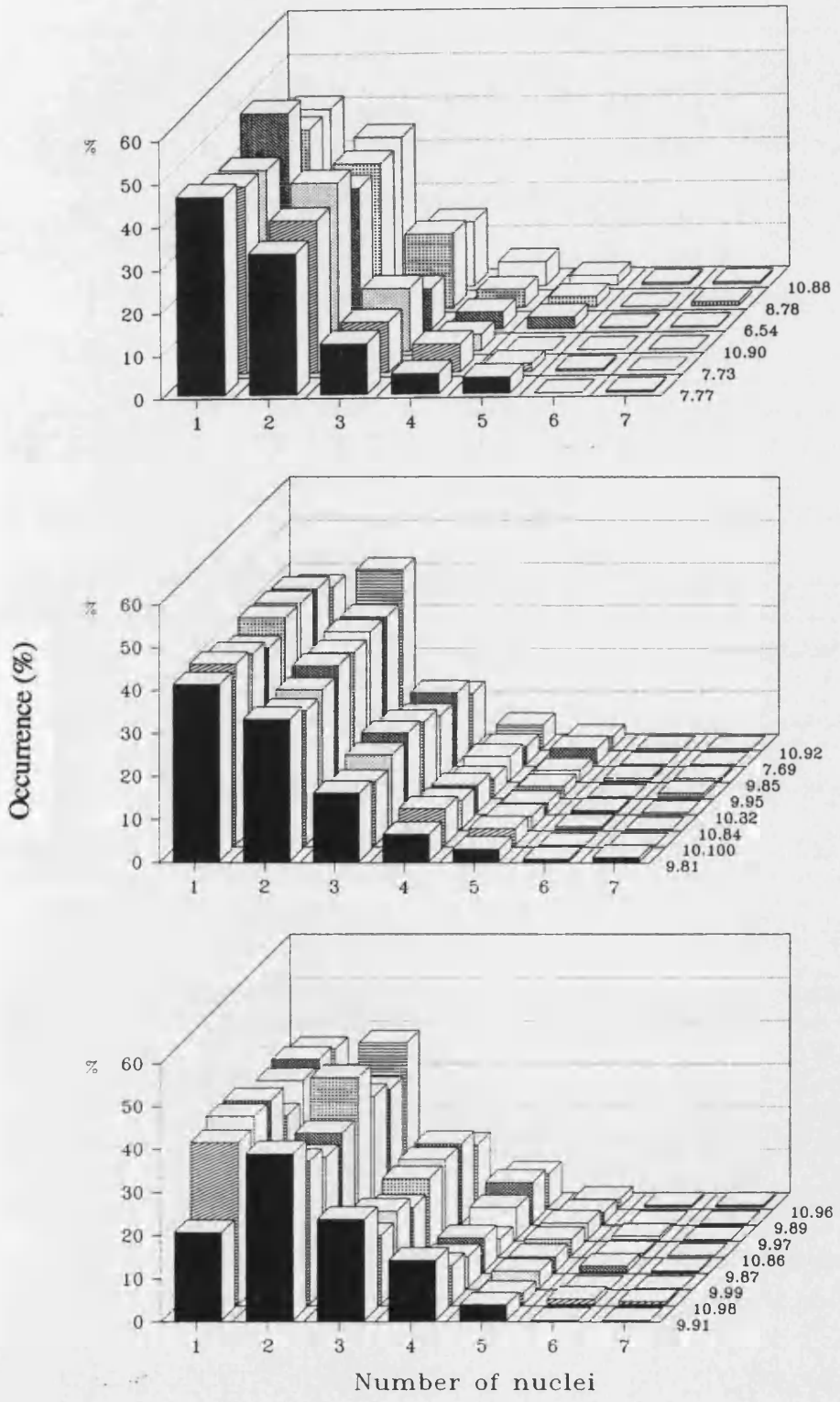
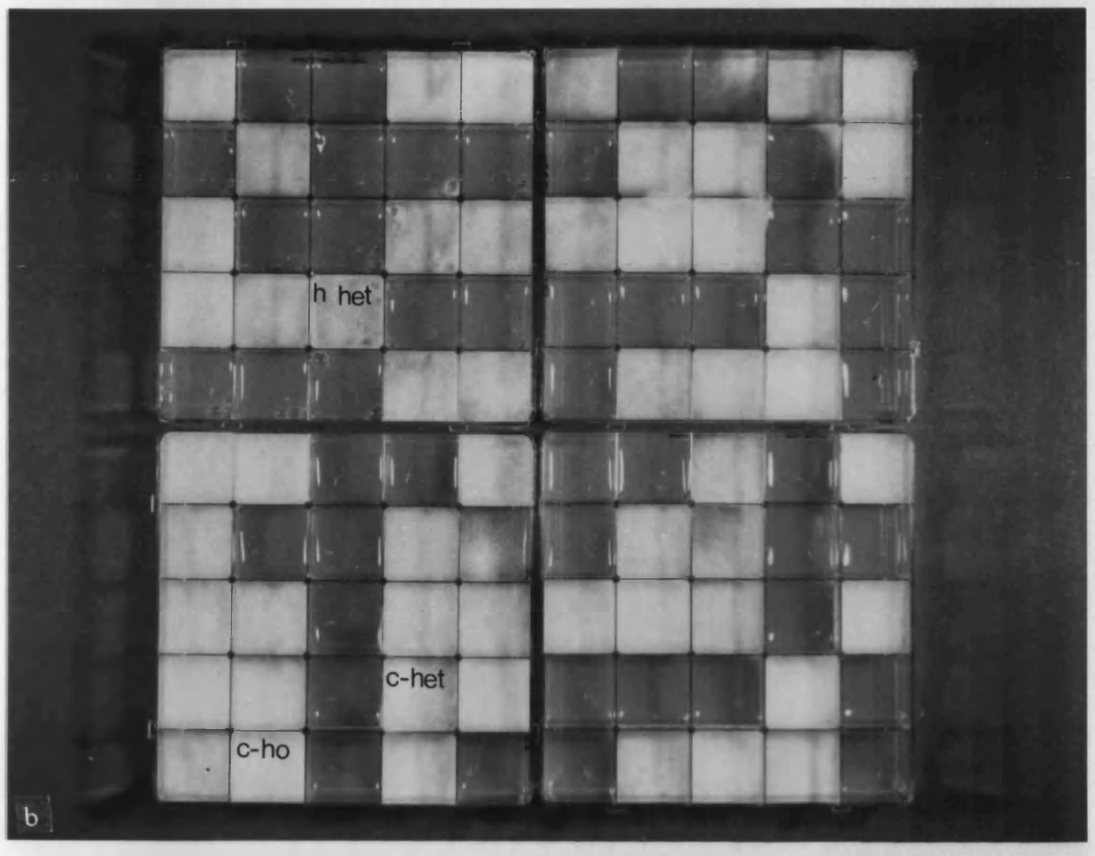


Table 5.2. *Recovery of homokaryotic and heterokaryotic genotypes from protoplasted progenitor homokaryons and 'hirsutum-complicatum' heterokaryons.*

Strain	*n*	H-type Homo (%)	H-type Het (%)	C-type Het (%)	C-type Homo (%)	PGM (%)	Phenotype of parent
EP18·7	97	100	0	0	0	3	H-ho (h)
10·100	87	84	8	0	8	6	H-het (h)
9·85	11	45	55	0	0	88	H-het (h)
9·95	28	39	57	0	4	69	H-het (h)
7·69	53	0	47	15	38	47	H-het (h)
10·90	54	0	9	43	48	45	H-het (h)
6·54	36	0	50	36	14	64	C-het (h)
9·83	71	0	65	8	27	27	C-het (c)
9·81	73	0	51	11	38	26	C-het (c)
10·86	63	0	32	21	48	36	C-het (c)
10·92	72	4	39	6	51	28	C-het (c)
8·78	72	0	29	18	53	23	C-het (c)
9·89	71	0	20	23	58	24	C-het (h)
9·99	62	3	18	19	60	22	C-het (c)
7·73	77	4	18	9	69	18	C-het (c)
10·82	63	0	6	19	75	20	C-het (c)
9·87	77	0	3	17	80	22	C-het (c)
9·97	91	0	0	3	97	9	C-ho (c)
JG1·5	95	0	0	0	100	5	C-ho (c)

Strain phenotypes are classified with regard to overall similarity to parental strains - (h) or (c) indicates side of original interaction plate from which source hyphal-tips were taken; *n*, number of protoplast strains recovered. PGM = post-germination mortality level.

Figure 5.4. Single protoplast derived isolates plated out onto MEA repli-dishes, (a) from a virtually homokaryotic strain 9.97; (b) from heterokaryon 10.90. Note the recovery of parental and non-parental phenotypes from the heterokaryon and the high level of post-germination mortality (blank squares).



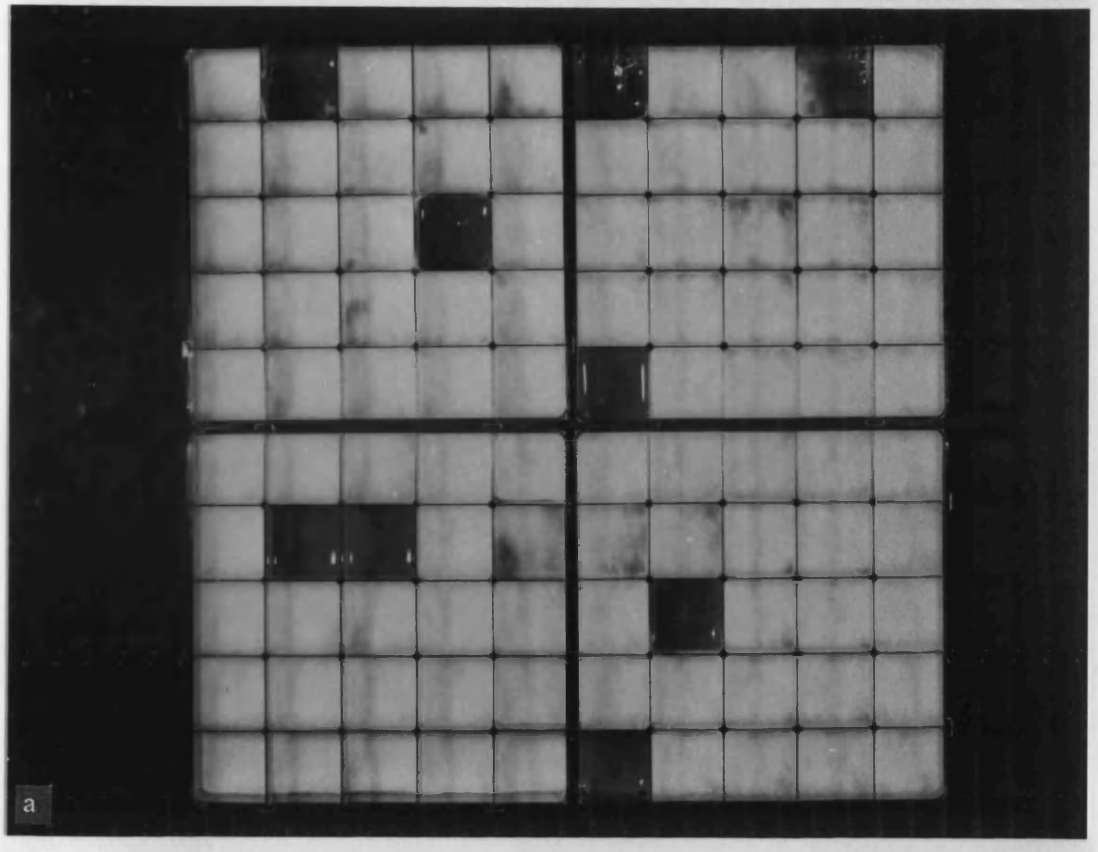
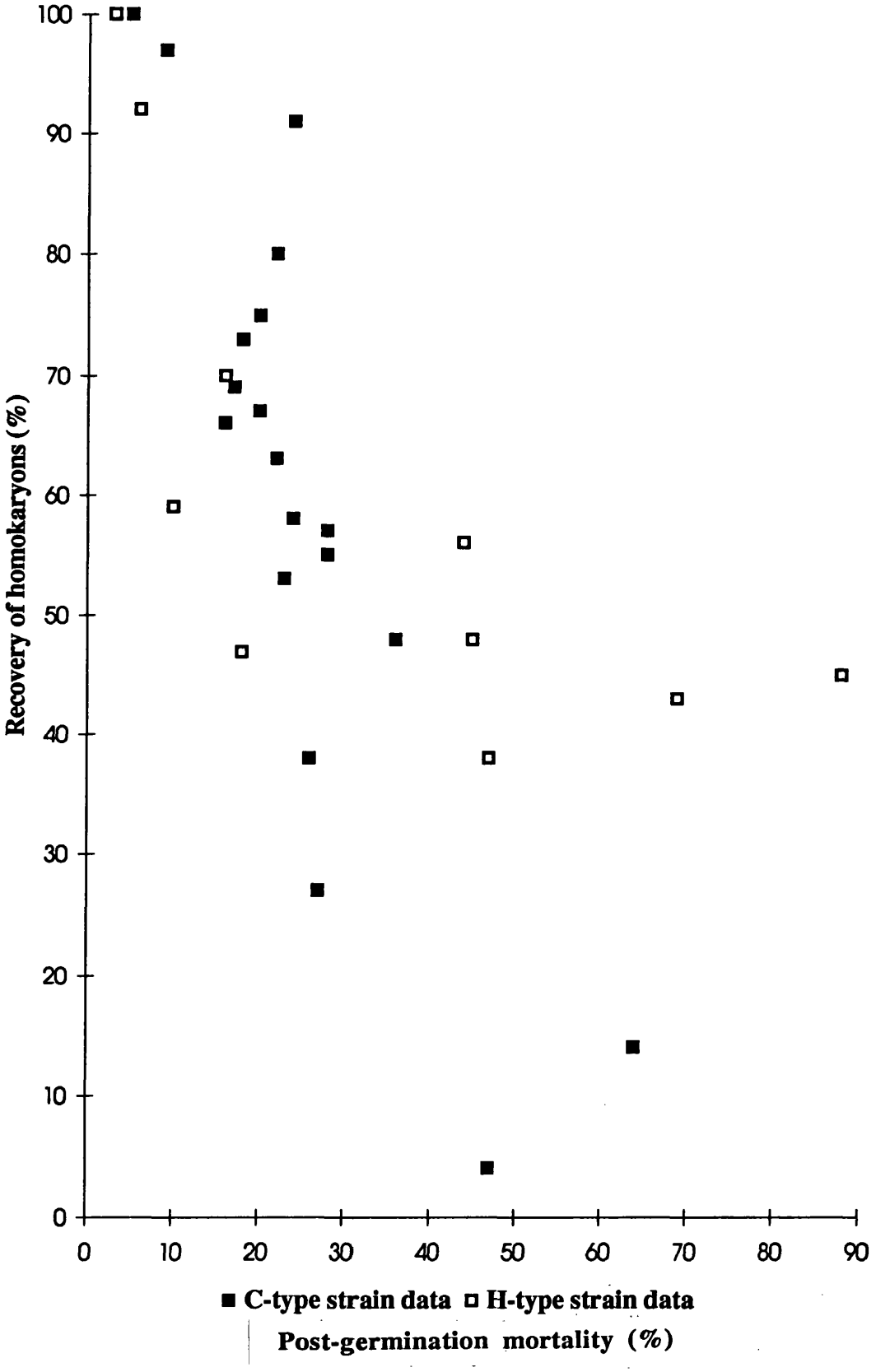


Figure 5.5. Graph relating post-germination mortality levels to the recovery of homokaryotic strains from '*hirsutum-complicatum*' heterokaryons. Original and 10.86 series data were combined (excluding the 'homokaryotic' outlier, 10.100), Pearson's product moment correlation coefficient, r (23 df), equals-0.693 (P<0.001). Indicating a high degree of correlation between these two variables.



heterokaryons, the increase in mortality occurred at a higher rate and in a substantially more non-linear fashion (r_p = -0.719, P<0.02).

Derived strain interactions

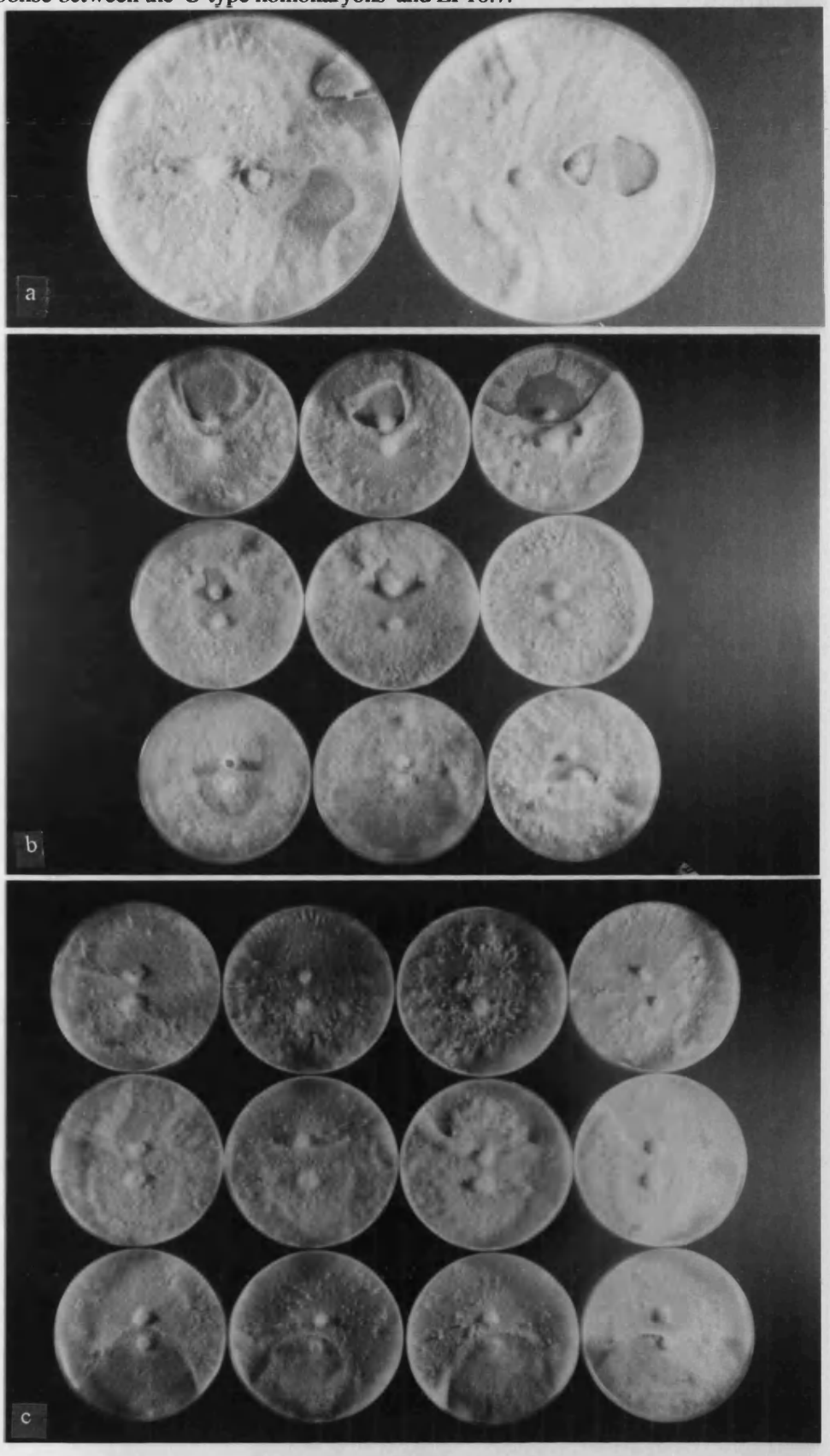
Pairings between the experimental heterokaryons and their progenitor homokaryons confirmed their identities and the descriptions of the strains given by Ainsworth *et al*, 1992. Pairings between C-type heterokaryons and EP18.7 also produced interaction patterns similar to those initially observed between the homokaryotic progenitor strains.

When H-type heterokaryons were paired with EP18.7, a spectrum of patterns were observed. At one extreme (eg EP18.7 x 10.90), a strong initial rejection response was followed by the production of an invasive 'bow-tie' shaped region with a *complicatum*-like phenotype in the domain previously occupied by the *hirsutum* homokaryon. Alternatively, as when EP18.7 was paired with 10.100, the initial rejection response was apparently followed by the conversion of the heterokaryon into a substantially more *hirsutum*-like mycelium. An apparent invasion of the heterokaryon by *hirsutum* nuclei from the homokaryon was suspected when protoplast regenerants obtained from this region produced a greater proportion of *hirsutum* homokaryons than the progenitor heterokaryon.

All C-type heterokaryons produced weak rejection responses against the *complicatum* parental strain. Pairings between H-type heterokaryons and JG1.5 resulted in the invasion of the heterokaryon by the *complicatum* genome, transforming heterokaryons into C-type colonies. In contrast to all of the other H-type pairings examined, strain 10.100 rejected JG1.5 and did not exhibit any morphological changes.

Patterns of interaction between protoplast-derived strains and their progenitor heterokaryons and parental homokaryons were readily categorized. *Complicatum* homokaryons paired against JG1.5 merged imperceptibly without any demarcation, but usually produced typical *hirsutum-complicatum* interactions when paired with EP18.7. However in some pairings, most notably involving *complicatum* homokaryons from 10.90, but also some from 10.82, the *complicatum* genome invaded more rapidly than usual and transformed EP18.7 directly into a *complicatum*-like phenotype (Figure 5.6). In pairings between protoplast-derived *complicatum*

Figure 5.6. 'Back-crossed' protoplast derived strains obtained from '*hirsutum-complicatum*' strain, 10.90. (a) H-type homokaryons paired against JG1.5; (b) Protoplast recovered heterokaryons paired against JG1.5 - top, EP18.7 - bottom and, heterokaryon 10.90 - bottom; and (c) C-type homokaryons from 10.90 paired against JG1.5 - top, EP18.7 - middle, and against heterokaryon 10.90 - bottom. Note particularly the absence of a significant rejection response between the 'C-type homokaryons' and EP18.7.



strains and their progenitor heterokaryons, morphological changes usually occurred in the heterokaryon which were suggestive of further invasion by *complicatum* nuclei.

For those pairings involving protoplast-derived C-type heterokaryons, patterns of interaction with the parental homokaryons were identical to those previously described. In several cases, when the derived heterokaryons were paired with their parental heterokaryons, complicatum genomes from the protoplast-derived strains invaded the parental strains. This was particularly evident when the parents were H-type heterokaryons.

Protoplast-derived *hirsutum* homokaryons were only available from five strains. They merged imperceptibly with both EP18.7 and with their progenitor heterokaryons; ie no somatic incompatibility was apparent. Pairings with JG 1.5 produced typical *hirsutum-complicatum* interactions.

Pairings involving protoplast-derived H-type heterokaryons exhibited the most complexity and variation. H-type heterokaryons derived from 7.69, when paired with the parental heterokaryon resulted in direct conversion of the derived strain into a C-type heterokaryon. The remaining strains merged with their progenitor heterokaryons without demarcation. In most pairings with *complicatum* homokaryons, derived H-type heterokaryons were invaded and converted to C-type heterokaryons. However, H-type heterokaryons derived from strains 10.90 and 10.100 both behaved as homokaryons when paired against JG1.5. With the exception of the H-type heterokaryons derived from 10.100, pairings with the *hirsutum* homokaryons produced consistent patterns. *Complicatum* nuclei from the H-type heterokaryon slowly invaded the homokaryotic progenitor, producing sienna-olivaceous 'bow-tie' shaped regions of appressed mycelium. These were bounded at their periphery by homokaryotic *hirsutum*-like growth. Later observations revealed that *complicatum* nuclei were present in this *hirsutum*-like region. Unlike other similar pairings, the 'bow-tie' shaped region was not transformed into a *complicatum*-like phenotype. The H-type derived heterokaryons from 10.100 rejected the *hirsutum* progenitor and no further interactions took place. Some examples of these altered interaction patterns are presented in Figure 5.6.

Altered recovery patterns

Positions of samples taken after the pairing of protoplast-derived H-type heterokaryon E4 with its parental homokaryotic and heterokaryotic strains are shown in Figure 5.7. The frequency of recovery of different colony types from the sample sites (Table 5.3), suggests that *complicatum* genomes are both more invasive and dominant than *hirsutum* genomes.

Heterokaryon E4, and its parental heterokaryon 10.86 did not differ significantly in genotype recovery patterns before pairing. With only one exception, samples taken from locations occupied by the pre-established heterokaryons (E4 or 10.86) did not demonstrate any significant changes in genotype frequency after pairing (Table 5.4).

When E4 was paired against the *complicatum* homokaryon a significant increase in the numbers of *complicatum* genotypes recovered from the E4 domain was observed for samples taken distal to the interaction interface. This implied that nuclei from the homokaryon had invaded the heterokaryon, skewing the nuclear ratios in favour of the *complicatum* genotype. A sample taken from a *hirsutum* like 'bow-tie' shaped region of mycelium close to the interaction interface surprisingly demonstrated no deviation from the original genotype recovery ratios, suggesting that the initial increase in contribution by *complicatum* was probably unstable. Remarkably in this case, the 'bow-tie' region was transformed into a C-type heterokaryon as time passed with no further changes in the genotypic ratios.

Genotype recovery patterns from the *hirsutum* side of the pairing between E4 and EP18.7 were particularly revealing. Large numbers of homokaryotic and heterokaryotic H-type colonies were recovered from sample position (8), which phenotypically still resembled the *hirsutum* homokaryon. However, the pattern of genotype recovery clearly demonstrated an invasion of the homokaryon by *complicatum* nuclei donated from the heterokaryon. At sample point (6), a portion of the mycelium exposed to invasion for longer, the *complicatum* genome became numerically better established. Moreover, no *hirsutum* homokaryons were recovered, suggesting that as a result of *complicatum* take-over, *hirsutum* genomes suffered a massive loss in viability. Isolated pockets of mycelium dominated by *hirsutum* genotypes did however remain at the margin of the interaction.

Figure 5.7. Source plates for examination of genotype recovery from 'back-crossed' 10.86 strains showing location of subsamples. (a) pairing between complicatum homokaryon, JG1.5, and derived heterokaryon, E4, showing conversion of the heterokaryon into a C-type morphology; (c) pairing between the progenitor '*hirsutum*' homokaryon, EP18.7 and derived heterokaryon E4; and (b) pairing between the original C-type heterokaryon, 10.86 and the derived H-type heterokaryon, E4, showing the conversion of the H-type heterokaryon into a degenerate morphology as the complicatum nuclei from the 10.86 heterokaryon invade.

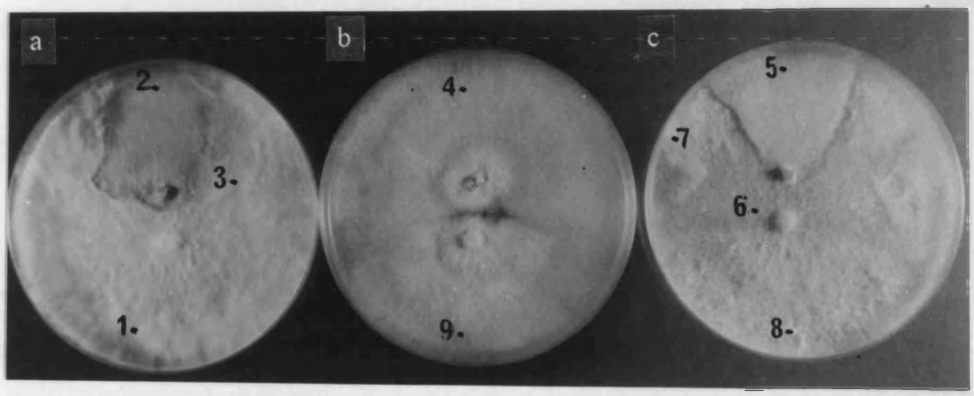


Table 5.3. *Genotype recoveries from heterokaryons generated from 'back-crossing' hirsutum-like single protoplast strain, E4 (derived from heterokaryon 10·86) with the homokaryotic parental strains, and the progenitor C-type heterokaryon, 10·86.*

Strain	*n*	H-type Homo (%)	H-type Het (%)	C-type Het (%)	C-type PGM Homo (%)	(%)	Side of origin
10·86	63	0	32	21	48	36	C-het*
E4	81	0	16	14	70	16	H-het*
HC 1	80	0	20	11	69	17	(JG1·5)
HC 2	74	0	5	4	91	24	(E4)
HC 3	75	0	23	11	67	20	(E4)
HC 4	82	0	17	17	66	16	(E4)
HC 5	66	0	26	17	57	28	(E4)
HC 6	81	0	37	16	47	18	(EP18·7)
HC 7	50	0	96	0	4	47	(EP18·7)
HC 8	63	34	44	0	22	44	(EP18·7)
HC 9	83	0	17	24	59	10	(10·86)

* Refers to phenotype of strains rather than origin in these cases. *n*, number of protoplast strains recovered.

Table 5.4. *Chi-squared, χ^2 (3 df), comparisons between genotype recoveries from the original C-type, 10·86 heterokaryon and the back-crossed H-type, E4 heterokaryon series.*

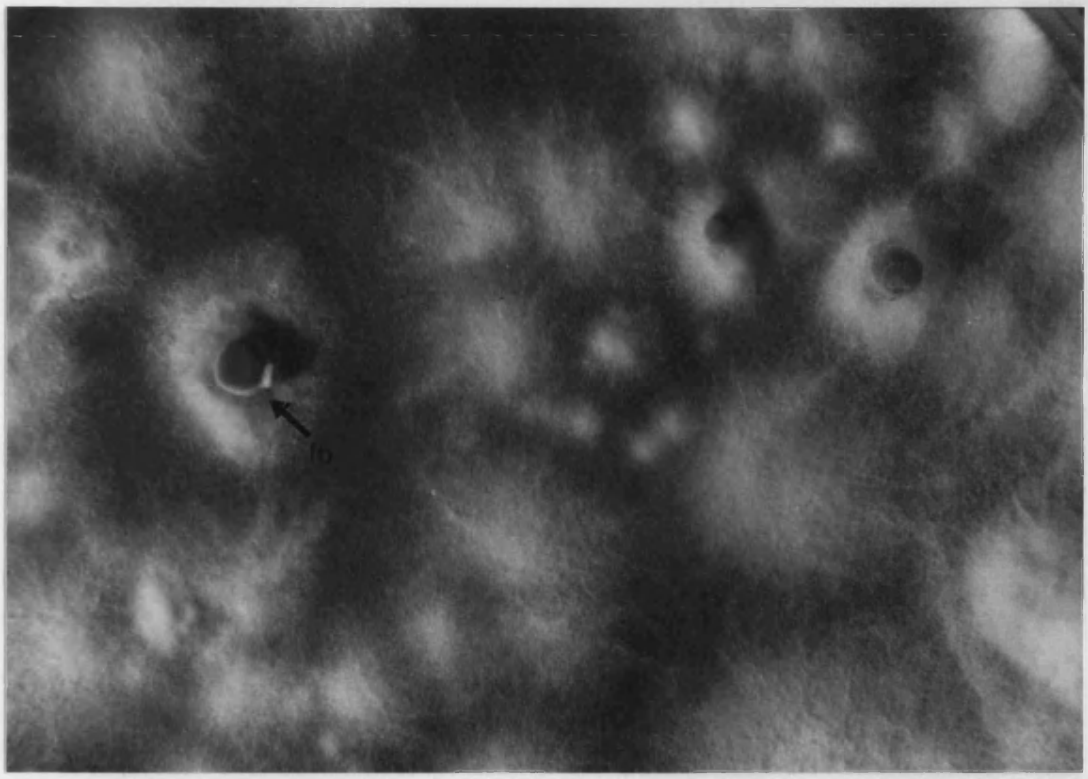
Strain x	Strain	χ^2	P<	Strain	χ^2	P<
E4	E4	0	nsd	10·86	7.904	*
10·86	E4	7.904	*	10·86	0	nsd
HC 1	E4	0.540	nsd	10·86	6.597	*
HC 2	E4	9.846	**	10·86	30.32	***
HC 3	E4	1.236	nsd	10·86	5.431	nsd
HC 4	E4	0.472	nsd	10·86	5.558	nsd
HC 5	E4	2.832	nsd	10·86	1.282	nsd
HC 6	E4	12.98	***	10·86	1.044	nsd
HC 7	E4	79.47	***	10·86	48.17	***
HC 8	E4	86.11	***	10·86	66.55	***
HC 9	E4	3.229	nsd	10·86	4.457	nsd
HC 4	HC 9	1.296	nsd			
HC 2	HC 5	20.24	***			
HC 3	HC 6	7.697	*			
HC 7	HC 8	66.77	***			

P < *, 0.050; **, 0.010; ***, 0.001; nsd, not significantly different

Fruitbody production

Fruitbody production was relatively rare on heterokaryotic mycelia plated out directly onto MEA or on interaction plates. Spread plates supporting dilute suspensions of regenerating protoplasts (500 colonies per plate or less) reliably produced 'plate' like or funnel shaped fruitbodies (Figure 5.8). Plates spread with higher concentrations of protoplasts (5000 or more) never produced fruitbodies. Furthermore in this situation, the developing mycelium was mostly submerged, degenerate and virtually lacked aerial mycelium.

Figure 5.8. Fruitbody production by '*hirsutum-complicatum*' protoplast spread plates. Plates kept at room temperature for 4 wk in natural light cycle.



DISCUSSION

Overall the patterns of genetic exchange and phenotypic variability observed in this study were consistent with those described in an earlier paper by Ainsworth *et al* (1992). Additional observations made here give further information pertaining to the complex relationship between the phenotype of an organism and its genotype; as well as the importance of past-history in modifying this relationship. Moreover, these observations provide an insight into the importance of intergenomic conflict within a cell, and hint at how organisms with indeterminate life-forms are capable of alleviating post-fusional incompatibilities.

With regard to the interplay between the genotype of an organism and its ultimate phenotype, several observations made during this study are of relevance. The recovery of mycelia with four distinct morphologies; two parental and two non-parental from *hirsutum-complicatum* heterokaryons is somewhat unexpected, particularly if no cytoplasmic exchange occurs during the establishment of a basidiomycete heterokaryon (Ainsworth *et al.*, 1992; Casselton & Economou, 1985 - but see next section). Surprisingly, the frequency of the four mycelial types recovered also varied between source heterokaryons with identical morphologies. For example, most of the H-type heterokaryons gave rise to regenerants that were mostly H-type, but two strains failed to give any H-type homokaryons whatsoever (producing only a few H-type heterokaryotic regenerants, and a higher proportion of C-type homokaryons and heterokaryons than some C-type source heterokaryons). At the other extreme, one strain (9.97), originally classified as a C-type homokaryon by Ainsworth *et al* (1992) on the basis of morphological, mating and molecular characteristics, was shown by protoplast recovery analysis to support *hirsutum* nuclei - otherwise it would not have produced C-type heterokaryons. The corollary of these observations is that in *Stereum*, the genetic constitution of a mycelium may not always be fully coupled with its phenotype.

How, then, can apparently identical genetic partnerships produce different phenotypes? Several explanations might account for this in *Stereum* heterokaryons; some that are purely genetic, and some that require the operation of epigenetic or hyperepigenetic processes (Rayner *et al.*, 1995).

The simplest genetic explanations involve the possible occurrence of asymmetric nuclear ratios within the heterokaryotic mycelia. As discussed previously, such asymmetries have been reported in a large number of fungal heterokaryons, but their importance as determinants of phenotype in this study are far from clear.

Single protoplast regenerants from *hirsutum-complicatum* heterokaryons displayed extremely high levels of post-germination mortality. This invalidates one of the assumptions required to estimate nuclear ratios from the frequency of recovery of regenerants; ie the components of a heterokaryon must not exhibit significant differential viability as a result of their past associations. It is therefore difficult categorically to assess the role that numerical dominance *per se*, plays in the determination of *hirsutum-complicatum* heterokaryon phenotype. However, as in the earlier studies of allopatric heterokaryons of *Heterobasidion annosum*, heterokaryotic *hirsutum-complicatum* mycelia often resembled the numerically or perhaps functionally subordinate parent. The data in Table 5.3 support this, revealing that when *complicatum* nuclei from an H-type heterokaryon invaded H-type mycelia, the phenotype remained substantially more *hirsutum*-like, despite the reduced viability of the *hirsutum* genome.

A second explanation might involve some form of epigenetic differentiation amongst nuclei within heterokaryons, leading to the over expression or suppression of one component genotype. Silencing might arise as a result of the activity of global repressors, perhaps operating in a manner analogous to the X-chromosome inactivation centre, *Xist*, of mammals (Brown *et al.*, 1991; Brown, 1992). Alternatively, silencing might occur if one of the genomes was damaged as a result of parasexual events triggered during the association. Such a possibility is supported by a number of observations made in this study and elsewhere.

Partial loss of genetic information from the *hirsutum* genome in *hirsutum-complicatum* heterokaryons was demonstrated by Ainsworth *et al*, (1992). The *hirsutum* genome was found to exist in one of three states which may be termed *complete, transitional* or *rudimentary.* Complete genomes contained all of the RFLP markers associated with the *hirsutum* parental strain, rudimentary genomes were abbreviated, and contained a smaller number of *hirsutum*

specific markers, whilst transitional genomes were intermediate, exhibiting variable degrees of genetic loss.

Very few H-type colonies were recovered in this study, reaffirming that *hirsutum* nuclei were less robust than their *complicatum* counterparts. An examination of the post-germination mortality data also strengthens the conclusion that *hirsutum* nuclei were disabled by their association with *complicatum* nuclei. Moreover, when the data on the rates of recovery of different phenotypes were linked to the levels of PGM, not only was the viability of the *hirsutum* genome decreased when associated with *complicatum* nuclei (to the extent that it was largely non-competent on its own), the decrease in viability also extended to the heterokaryotic association that it maintained.

When the data from Table 5.2 were transposed onto a map (AM Ainsworth personal communication) of the original pairings made by Ainsworth *et al* (1992) some clear patterns emerged. If the positions of the subcultures are ranked with respect to the abundance of either *hirsutum* or *complicatum* homokaryons, a random distribution of rankings (for 17 sample locations) should give a mean value of 9, with deviations from this indicating a non-random pattern. The mean ranking for the C-type domain was 9.0, for the H-type region 12.3 and for the interfacial region 6.1. Interfacial zones therefore supported the highest levels of non-self nuclei, the direct radial routes of access exhibited the next level of penetration, and peripheral or 'bow-tie' regions, supported the lowest levels. These patterns comply well with the acceptor-access-stablilization model of heterokaryon establishment proposed by Rayner *et al* (1984).

A high level of homokaryon recovery generally correlated with a low level of inviability. The rankings therefore indicate that the least damage occurred to nuclei in the interfacial zone, that there was very limited damage to nuclei in the *complicatum* region, and that most damage occurred in the *hirsutum* domain. Furthermore this held true regardless of the time during heterokaryon establishment that the subcultures were taken. Similar conclusions were reached when the levels of PGM were mapped onto the regions occupied by the original strains during heterokaryon formation. PGM was lowest in the interfacial zone itself (mean rank 5.2), average in the C-type domain (mean rank 9.5), and above average in the H-type domain (mean rank 12.0).

The most enlightening correlations concern the molecular status of the genomes (reported by Ainsworth *et al.*, 1992) and the level of PGM exhibited by regenerants. Rudimentary genomes had a mean ranking of 9.0, indicating that the level of PGM was random; complete genomes, with a mean ranking of 14.3 were associated with a lower than expected incidence of PGM; whereas transitional genomes, with a mean ranking of 4.2 were associated with a higher incidence. It may therefore be concluded that post-germination mortality is reduced when conflicting portions of genetic information are removed. Developmental incompatibilities expressed between nuclei may be alleviated by the selected or directed loss of genetic information - perhaps as a result of parasexual type events. Not surprisingly, the intermediate states between an intact genome and a rudimentary genome are highly unstable and are mostly non-competent. This agrees with the finding that partial diploids are often less viable than aneuploids or haploids (Käfer, 1960; Raper & Flexer, 1970).

Free-radical and reactive oxygen species generation is regarded as a potentially important component of the breakdown of unstable heterokaryons (Rayner, 1994b, 1996). Such agents might also account for the damage observed to occur in *hirsutum* nuclei. Free-radical induced DNA-damage can act as a stimulus initiating DNA repair mechanisms that lead to chromosomal loss. Evidence for such a mechanism in fungi has been provided by the finding that UV-light, mitomycin and fluorodeoxyuridine initiate mitotic crossing-over and haploidization in the normally stable diploids of *Ustilago maydis* (Holliday, 1961,1964; Easton & Holliday, 1964).

Additional data provided in this study appear to indicate that nuclei from a homokaryon could invade an established heterokaryon. However, this evidence is not direct, but is inferred from the increase in the recovery of parental genotypes from heterokaryons that were backcrossed with parental homokaryons. Several alternative explanations are feasible. Firstly, nuclei from the homokaryon may have invaded the heterokaryon increasing their level of representation. Secondly, during such as invasion process, nuclei with a mating-type complementary to that of the homokaryon (equivalent to the resident nuclei in a normal mating), suffered a loss of viability so that they were not viable. Lastly, nuclei from a heterokaryon with a mating-type complementary to the homokaryon, might have invaded the homokaryon without replicating,

thereby leaving behind fewer numbers of nuclei in the source heterokaryon. Work by Snider (1968) has shown that the migrating nuclei in di-mon matings do not divide during an invasion, lending some support to the latter suggestion.

The direct conversion of established heterokaryons to a new phenotype during these back-crosses has a considerable bearing upon our understanding of phenotype-genotype relations. For example in pairings between C-type homokaryons and H-type heterokaryons, the H-type heterokaryon was often converted to a heterokaryon with a C-type morphology. Similarly, in pairings between two heterokaryons, the *complicatum* nuclei often appeared to invade the H-type heterokaryons, converting them to a C-type morphology. Some of the results obtained however, were more equivocal. For example in one instance, two H-type heterokaryons paired together produced a C-type heterokaryon (eg 7.69 x H-type regenerant).

Examination of the genotype recovery data for the last pairing revealed that these strains supported unusually high proportion of C-type nuclei which easily accounts for the final outcome (but not the original appearance of the heterokaryons). Changes in nuclear ratios within mycelia might account for the observed patterns of phenotypic emergence observed in some of the other pairings. For example, analyses of nuclear ratios in the 18.7 x 10.100 pairing indicated that to a large extent that the final outcome did reflect the total contribution of each genotype to the heterokaryon that emerged. However, as already established, this was not always the case.

Another possibility, association-specific-priming or acclimation, is highlighted by the pairings between H-type heterokaryons and the *complicatum* progenitor strain or C-type regenerants. Here the original heterokaryons rapidly expressed C-type morphologies, suggesting that the *hirsutum* nuclei within the heterokaryons were primed for further invasion by *complicatum* nuclei. Such a process of acclimation might involve epigenetic modifications or imprinting, or alternatively the loss of conflicting information as already described. The consequences of acclimation might include; (i) increased rates of invasion (a pattern which was detected), (ii) decrease incompatibilities during further interactions (cf behaviour of *H.annosum* derived homokaryons), or (iii) more rapid stabilization.

Hurst (1991c), proposed that when matings between allopatrically derived populations occur, various intracellular 'spiteful' symbionts are brought into close proximity. If the 'spiteful' symbionts are different this may result in bidirectional incompatibility. Conversely, when only one population contains a spiteful symbiont, the matings would only be unidirectionally incompatible. Hurst envisaged that the symbionts involved were the ricketssid-like prokaryotes, spirochaete-like bodies, or virus-like particles which cause post-fusional incompatibilities in some insects.

The complex patterns of heterokaryon establishment following interactions between *hirsutum* and *complicatum* mycelia (with apparent stability on the *complicatum* side and instability on the *hirsutum* side), as well as the occurrence of post-germination mortality amongst protoplast regenerants, show some similarity to the patterns of hybrid sterility observed when mosquitoes from diverse geographical locations are interbred (Breeuwer & Werren, 1990; O'Neill & Karr, 1990).

Similar elements to those responsible for hybrid sterility in insects have been described in a number of fungi (Markham, 1995). No evidence for them has been found in *Stereum*, (see next section), but the isolates examined were not those used in this particular study and so this possibility cannot be entirely ruled out.

SECTION VI

PATTERNS OF MITOCHONDRIAL INHERITANCE AND ALTERED SOMATO-SEXUAL RECOGNITION PROPERTIES IN HOMOKARYONS AND HETEROKARYONS OF STEREUM HIRSUTUM.

INTRODUCTION

In addition to nuclei, fungal cytoplasms typically contain mitochondria, vesicles, vacuoles and an assortment of microbodies typical of most eukaryotes. They may also contain inclusions with a more restricted taxonomic distribution, such as woronin bodies, hexagonal crystals, hydrogenosomes, lomasomes / multivesicular bodies, rumposomes and gamma particles (Markham, 1995). Some of these organelles or cytoplasmic inclusions possess their own genetic information, and might potentially be in conflict with each other, or with their nuclear partners. Uniparental organelle inheritance has been proposed to occur within many eukaryotes as a means of reducing these intra- and / or inter-genomic conflicts (Eberhard, 1980; Cosmides & Tooby, 1981, see also Rayner & Ross, 1988).

The importance of genomic conflict becomes particularly apparent when interspecific and intergeneric hybrids are examined. For example, hybrids between angiospermous plants exhibit cytoplasmic male sterility at a very high frequency (Darwin, 1877; Laser & Lersten, 1972; Frank, 1989). It appears that when cytoplasmic male sterility genes invade a population they rapidly spread to fixation. Concomitantly, nuclear suppressors to specific cytoplasmic genes also carry to fixation, masking the effect of the original variant. When a nuclear genome is faced with a cytoplasmic male sterility factor from another species, there may be no co-evolutionary countermeasure present, consequently the hybrids are often sterile. Other cytoplasmic variants are known that affect such diverse aspects of organismal biology as the allocation of reproductive effort in hermaphrodites (Rhoades, 1933) and the sex ratios of offspring (Poulson, 1963,1968).

A variety of potentially infectious selfish genetic elements are located within the cytoplasms of fungi. These include nuclear plasmids, linear and circular mitochondrial plasmids, viruses and virus-like particles (Clutterbuck, 1995), many of which are capable of affecting the phenotype of the mycelium in which they reside (Thoday, 1958; Jinks, 1959; Srb, 1958,1963; Wood & Bozarth, 1973; Buck, 1980; Gunge, 1983 in Rogers, Buck & Brasier, 1986).

When considering the conflicts that may occur following the establishment of a new genetic partnership within a heterokaryon, it is clearly important to understand what patterns of

cytoplasmic inheritance are possible, what elements are involved and how these may affect the outcome of intergenomic interactions.

As discussed in the previous section, complex patterns of nuclear and mitochondrial inheritance have been revealed within various members of the genus *Stereum* (Coates & Rayner, 1985a,b; Beeching *et al.*, 1989; Ainsworth *et al.*, 1990a, 1992). Moreover, a curious relationship has been observed between some combinations of *Stereum hirsutum* isolates that blurs the distinction between nuclear and cytoplasmic determinants of self-non-self recognition and incompatibility (Coates & Rayner, 1985c).

A dramatic change in the somatic behaviour and sexual recognition responses of a single homokaryotic strain of *Stereum hirsutum* was reported by Coates & Rayner (1985c). The altered properties of the mycelia were transmissible in both compatible and incompatible matings, apparently without nuclear transfer. The inheritance of the altered state was thought to be under the control of a cytoplasmically transmissible 't-factor'.

The present study reports further on the inter-relationships between nuclear and mitochondrial genomes in the determination of somato-sexual responses in a variety of normal and 'transformed' (t-factor-containing) strains of *Stereum hirsutum*.

MATERIALS AND METHODS

Strains and subculturing

Unless otherwise stated all strains were grown at 20°C in 90 mm diameter Petri dishes containing approximately 15 ml MEA. Stock-cultures were maintained at 4°C on MEA bijou slopes under sterile mineral oil.

Homokaryotic and heterokaryotic strains of *Stereum hirsutum*, derived from naturally-occurring and laboratory-induced fruitbodies, were taken from the University of Bath culture collection. The strains examined reflect a range of genetic constitutions, geographical backgrounds and genomic relations. Individual strain designations, origins and source references are given in Table 6.1. More detailed information about the strains examined can be found in the references cited.

Many strains were subjected to a preliminary nuclear and mitochondrial DNA fingerprinting screen to determine both the extent of the variation present, and to assess which markers were suitable for following nuclei or mitochondria through matings (Table 6.2).

Initial pairings

In order to ascertain cultural variability, mating-type specificity and ability to form 'bow-ties' (Coates, Rayner & Todd, 1981; Coates & Rayner, 1985b), pairings were set up in all possible combinations between working cultures of the F2 basidiospore progeny series. Working cultures were regularly subcultured onto MEA plates at 20°C and then back onto MEA bijou slopes before being stored under oil at 4°C. Following the initial pairings, more pairings were set up between 'old' F2 progeny series strains that had been maintained continuously under oil at 4°C.

'Old' strains of F2·3-F2·19 were obtained that had remained in cold-storage under oil for 7 years, whilst strains of F2·101-F2·119 were found that had remained untouched for 5 years (the age quoted equates to the time elapsed since isolation from an F2 fb culture that fruited in the laboratory - Martyn Ainsworth, personal communication).

For all pairings, inoculum plugs, 6 mm in diameter were cut from the edges of 7-d-old cultures and were placed 1 cm apart in the centre of MEA plates. The results of pairings were compared to those reported by Coates, Rayner & Todd (1981) and Coates (1984).

Table 6.1. *Designations and origins of Stereum strains examined*

Strain	Collector	Substratum	Source	Origin	Refs
F2 fb	DC	*Quercus*	fb	Somerset, UK	(1)(2)(4)
F2·3	DC*	*Quercus*	bsfb	Somerset, UK	(1)(4)
F2·4	DC	*Quercus*	bsfb	Somerset, UK	(1-5)
F2·4t	DC	*Quercus*	myc	Lab strain	(2-5)
F2·4rt	AMA	*Quercus*	myc	Lab strain	(2)
F2·4rnt	AMA	*Quercus*	myc	Lab strain	(2)
F2·9	DC	*Quercus*	bsfb	Somerset, UK	(1)(2)(4)
F2·9t	DC	*Quercus*	myc	Lab strain	(2)
F2·9nt	AMA	*Quercus*	myc	Lab strain	(2)
F2·10	DC	*Quercus*	bsfb	Somerset, UK	(1)(3)(4)
F2·11	DC*	*Quercus*	bsfb	Somerset, UK	(1)(4)
F2·12	DC*	*Quercus*	bsfb	Somerset, UK	(1)(4)
F2·13	DC*	*Quercus*	bsfb	Somerset, UK	(1)(4)
F2·18	DC*	*Quercus*	bsfb	Somerset, UK	(1)(4)
F2·19	DC*	*Quercus*	bsfb	Somerset, UK	(1)(4)
F2·105	AMA**	*Quercus*	bslab	Somerset, UK	(1)
F2·106	AMA**	*Quercus*	bslab	Somerset, UK	(1)
F2·108	AMA**	*Quercus*	bslab	Somerset, UK	(1)
F2·111	AMA**	*Quercus*	bslab	Somerset, UK	(1)
F2·112	AMA**	*Quercus*	bslab	Somerset, UK	(1)
F2·113	AMA**	*Quercus*	bslab	Somerset, UK	(1)
F2·114	AMA**	*Quercus*	bslab	Somerset, UK	(1)
F2·119	AMA**	*Quercus*	bslab	Somerset, UK	(1)
F2·101	AMA	*Quercus*	bslab	Lab strain	(1)
F2·102	AMA	*Quercus*	bslab	Lab strain	(1)
F2·103	AMA	*Quercus*	bslab	Lab strain	(1)
F2·107	AMA	*Quercus*	bslab	Lab strain	(1)
F2·109	AMA	*Quercus*	bslab	Lab strain	(1)
F2·110	AMA	*Quercus*	bslab	Lab strain	(1)
F2·115	AMA	*Quercus*	bslab	Lab strain	(1)
F2·116	AMA	*Quercus*	bslab	Lab strain	(1)
F2·117	AMA	*Quercus*	bslab	Lab strain	(1)
F2·118	AMA	*Quercus*	bslab	Lab strain	(1)
AF1·6	DC	*Fraxinus*	bsfb	Somerset, UK	(1)

Source references: (1) Ainsworth & Rayner (1989) (2) Beeching *et al.* (1989) (3) Ainsworth *et al.* (1990) (4) Coates, Rayner & Todd (1981) (5) Coates & Rayner (1985a) (6) Ainsworth *et al.* (1992). (fb) = Fruitbody tissue isolate; (bsfb) = single basidiospore isolate from source fruitbody; (myc) = single hyphal-tip from mycelial interaction plate; (bslab) = single basidiospore isolate from laboratory-induced fruiting. Collectors were; (AMA) M Ainsworth, (PB) P Bayman, (DC) D Coates, (JG) J Ginns and (EP) E Parmasto.

Table 6.1 continued. *Designations and origins of Stereum strains examined*

Strain	Collector	Substratum	Source	Origin	Refs
EP1 fb	EP	*Carpinus*	fb	Caucasus, CIS	(1)
EP1·2	EP	*Carpinus*	bsfb	Caucasus, CIS	(1)
EP1·3	EP	*Carpinus*	bsfb	Caucasus, CIS	(1)
EP1·4	EP	*Carpinus*	bsfb	Caucasus, CIS	(1)
EP1·5	EP	*Carpinus*	bsfb	Caucasus, CIS	(1)
EP1·6	EP	*Carpinus*	bsfb	Caucasus, CIS	(1)(3)
EP1·7	EP	*Carpinus*	bsfb	Caucasus, CIS	(1)(2)
EP1·9	EP	*Carpinus*	bsfb	Caucasus, CIS	(1)
EP1·10	EP	*Carpinus*	bsfb	Caucasus, CIS	(1)
EP1·11	EP	*Carpinus*	bsfb	Caucasus, CIS	(1)
EP1·12	EP	*Carpinus*	bsfb	Caucasus, CIS	(1)
EP1·13	EP	*Carpinus*	bsfb	Caucasus, CIS	(1)
EP18·7	EP	*Populus*	bsfb	Ukraine, CIS	(1)
PB3·14	PB	*Umbellularia*	bsfb	California, US	(1)(3)(6)
JG1·5	JG	*Fagus*	bsfb	New York, US	(1)(6)
BR1 fb	AMA	*Quercus*	fb	Avon, UK	
BR2 fb	AMA	*Quercus*	fb	Avon, UK	
G2 fb	AMA	*Betula*	fb	Avon, UK	
NF1 fb	AMA	*Quercus*	fb	Hampshire, UK	
HH1 fb	AMA	*Quercus*	fb	Herts, UK	
U11 fb	AMA	*Corylus*	fb	Avon, UK	

Source references: (1) Ainsworth & Rayner (1989) (2) Beeching *et al.* (1989) (3) Ainsworth *et al.* (1990) (4) Coates, Rayner & Todd (1981) (5) Coates & Rayner (1985a) (6) Ainsworth *et al.* (1992). (fb) = Fruitbody tissue isolate; (bsfb) = single basidiospore isolate from source fruitbody; (myc) = single hyphal-tip from mycelial interaction plate; (bslab) = single basidiospore isolate from laboratory-induced fruiting. Collectors were; (AMA) M Ainsworth, (PB) P Bayman, (DC) D Coates, (JG) J Ginns and (EP) E Parmasto.

Strains F2.4 (C1 mating-type) and F2.9 (C1 mating-type) were obtained as single basidiospore isolates from a naturally occurring fruitbody (F2) by David Coates. Strain F2.4t was derived from a stock culture of F2.4 which had undergone a spontaneous change during storage, causing it to acquire, in the absence of mating, some of the characteristics of a heterokaryon. Strain F2.9t was generated by pairing F2.9 with F2.4t and then taking subcultures from the morphologically changed mycelium that developed within the F2.9 domain. F2.4rt was generated by back-pairing F2.4 with F2.9t and then taking sub-cultures from the F2.4 side of the interaction. Martyn Ainsworth repeated some of the original pairings to produce F2.9nt and F2.4rnt.

Table 6.2. *Strains of Stereum subjected to nuclear and mitochondrial DNA haplotype analysis*

Strains	Target DNA			Probe DNA		
	TG	N	mt	TG	N	mt
F2 fb	+	+	+ul	-	+	+
F2·4	+	+	+ul	-	+	+
F2·4t	+	+	+ul	-	-	+u
F2·4rt	+	+	+ul	-	-	-
F2·4rnt	+	+	+ul	-	-	-
F2·9	+	+	+ul	-	-	+
F2·9t	+	+	+ul	-	-	+
F2·9nt	+	+	+ul	-	-	-
F2·10	+	+	+ul	-	-	+
F2·101	+	-	-	-	-	+u
F2·102	+	-	-	-	-	+1,2,6,7
F2·103	-	-	-	-	-	+3
F2·107	+	-	-	-	-	+
F2·109	-	-	-	-	-	+9
F2·110	+	-	-	-	-	+
F2·115	+	-	-	-	-	+
F2·116	+	-	-	-	-	-
F2·117	+	-	-	-	-	+
F2·118	+	-	-	-	-	+
AF1·6	+	+	+	-	-	+
EP1 fb	+	-	-	-	-	+
EP1·2	-	-	-	-	-	+
EP1·3	-	-	-	-	-	+
EP1·4	+	-	-	-	-	+
EP1·5	-	-	-	-	-	+
EP1·6	-	-	-	-	-	+
EP1·7	+	-	-	-	-	+
EP1·9	-	-	-	-	-	+
EP1·10	+	-	-	-	-	-
EP1·11	+	-	-	-	-	+
EP1·12	+	-	-	-	-	-
EP1·13	+	-	-	-	-	-
EP18·7	+	+	+	-	-	+
PB3·14	+	+	+	-	-	+
JG1·5	+	-	-	-	-	+
BR1 fb	+	-	-	-	-	+
BR2 fb	+	-	-	-	-	+5,6,9-11
G2 fb	+	-	-	-	-	+
NF1 fb	+	-	-	-	-	+
HH1 fb	+	-	-	-	-	+
U11 fb	+	-	-	-	-	+

TG = total genomic DNA; N = purified nuclear DNA; mt = mitochondrial DNA; u = upper mitochondrial band from CsCl gradients; l = lower mitochondrial band from CsCl gradients. Numbers in final column indicate specific EcoRV fragments of mitochondrial DNA.

Pairings to examine mtDNA inheritance

Pairings were set up between homokaryotic strains of *S.hirsutum* with known mating-type specificities and mitochondrial DNA (mtDNA) haplotypes based on data from a preliminary screening. Some heterokaryon : heterokaryon pairings (He:He) and heterokaryon : homokaryon (He:Ho) pairings were also made. Pairings were set up as follows: AF1·6 x (F2 fb, F2·10, F2·101, F2·4t); EP1 fb x (F·2 fb, F2·10, F2·101, F2·4t) and EP1·7 x (F2 fb, F2·10, F2·101, F2·4t). After 4 wk growth, 20 small 'representative' fragments of mycelium were taken from various locations on the interaction plates and transferred to 0.02% MEA plates overlaid with sterile cellophane. Precise sampling positions were dictated by interaction morphologies rather than any pre-ordained random sampling regime. After 3-d growth, single hyphal-tips were excised according to the method of Butler (1984).

After an examination of their cultural characteristics, representative hyphal-tip cultures were subjected to molecular mtDNA haplotype analysis.

Isolation of total genomic DNA

Strains were grown for 7-d at 20°C, on 8 cm diameter sterile cellulose discs (350 POO, BCL Cellophane) overlying MEA. Mycelia from five plates per strain were harvested, homogenized and freeze-dried overnight. Total genomic DNA was extracted from the strains using a modification of the method described by Raeder & Broda (1985).

Powdered mycelium was suspended in chilled extraction buffer (200 mM Tris.HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and agitated for 30 min. Samples were treated once with an equal volume of phenol / chloroform / iso-amyl alcohol (25:24:1) and twice with chloroform / iso-amyl alcohol. DNA was precipitated with 0.6 volumes of isopropanol at -20°C and recovered by centrifugation at 13 000 rpm for 10 mins. Finally, DNA pellets were washed twice in 70% ethanol, vacuum-dried and resuspended in 10 mM Tris.HCl pH 7.5, 1 mM EDTA.

Isolation of mitochondrial DNA

Mitochondrial DNA samples were prepared in two ways; by direct extraction of DNA from purified intact mitochondria (Rogers, Buck & Brasier, 1987), and/or by CsCl buoyant density ultracentrifugation of total genomic DNA (Garber & Yoder, 1983).

Purification of mitochondria:

For isolation from intact mitochondria, mycelium from 20 plates per strain was harvested and transferred to 150 ml chilled 438 mM sucrose in buffer A (buffer A: 10 mM Tris.HCl pH 7.5, 0.02 mM EDTA). After 15 seconds maceration on a blender, the samples were made upto 250 ml with buffered 29 mM sucrose and the mycelial debris was pelleted twice by centrifugation at 3 500 rpm for 10 min (4°C). The supernatant was then centrifuged at 11 000 rpm for 30 min (4°C) to produce a crude mitochondrial pellet. The pellets were resuspended in 2.75 ml buffered 584 mM sucrose, and then layered onto a sucrose step gradient (upper phase = buffered 1 M sucrose; lower phase = buffered 1.75 M sucrose). After spinning the samples at 25 000 rpm for 90 min (4°C) a mitochondrial fraction was collected from the step gradient interface. Fractions were diluted with buffer B (buffer B: 10 mM Tris.HCl pH 8.0, 0.2 mM EDTA) and pelleted at 13 000 rpm for 30 min (4°C).

After removal of the supernatant, mitochondria were lysed in 50 mM Tris.HCl pH 8.0, 5 mM EDTA, 50 mM NaCl, 2% SDS. DNA was purified by a double extraction with phenol / chloroform / iso-amyl alcohol (25:24:1) and a single extraction with chloroform / iso-amyl alcohol (24:1). DNA was precipitated for 15 min at -80°C with sodium acetate, pH 5.2 (final concentration 150 mM) and an equal volume of absolute ethanol. Finally DNA samples were pelleted, washed in 70% ethanol, freeze-dried and dissolved in 10 mM Tris.HCl pH 8.0, 1 mM EDTA.

CsCl / bis-benzimide ultracentrifugation:

Mycelia from 120 plates per sample were harvested, homogenized and freeze-dried. Powdered mycelium (2-3 g) was agitated for 10 min at room temperature in 50 ml buffered 350 mM sucrose (buffer: 10 mM Tris.HCl pH 7.5, 50 mM EDTA) containing 20 $\mu g\ ml^{-1}$ proteinase K. Cellular debris was pelleted at 3 500 rpm for 10 min (4°C). The supernatant was filtered through sterile muslin and then centrifuged at 25 000 rpm for 30 min (4°C). The resulting crude nuclear / mitochondrial pellet was resuspended in 2.25 ml lysis buffer (lysis buffer: 10 mM Tris.HCl pH 7.5, 50 mM EDTA, 150 mM NaCl, 20 $\mu g\ ml^{-1}$ proteinase K, 2% SDS).

Lysis was promoted by gentle rocking at room temperature for 10 min and then incubation at 65°C for 40 min. Samples were chilled on ice prior to centrifugation at 7 000 rpm for 10 min (4°C). The supernatant was repeatedly extracted with phenol (equilibrated with 10 mM Tris.HCl pH 7.5, 50 mM EDTA) until no further debris formed at the organic / aqueous phase interface following centrifugation at 6 500 rpm for 10 min. The samples were then extracted once with phenol / chloroform / iso-amyl alcohol (25:24:1) and once with chloroform / iso-amyl alcohol (24:1).

Sample DNAs were separated at 40 000 rpm (20°C) for 24 h on CsCl gradients (initial density, $\rho = 1.666$ g ml^{-1}) containing 120 µg ml^{-1} bis-benzimide . Nuclear and mitochondrial DNA bands were visualized under short-wave UV light (254 nm) and removed separately by puncturing the centrifuge tubes with wide-bore syringe needles (19-gauge). Dye was removed from the DNA samples by repeated extraction of the samples with CsCl-saturated iso-propanol. When no further dye could be detected under UV light, the samples were diluted in 3 volumes of 10 mM Tris.HCl pH 7.5, 50 mM EDTA. DNA was precipitated with 0.05 volumes of 8 M LiCl and 2 volumes of absolute ethanol at -20°C for 2 h and then pelleted at 10 000 rpm for 30 min. DNA samples were finally dissolved in 10 mM Tris.HCl pH 7.5, 50 mM EDTA.

rDNA amplification

16S rDNA was amplified using the NS1 and NS8 primers of White *et al* (1990) or the A and B primers of Sogin (1990). For standard amplifications, PCR was carried out using 100 ng total genomic target DNA in the following reaction mixture; 10 mM Tris.HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatine, 1.0 mM each dNTP, 2 µM primer, 5 units Taq polymerase. Reaction conditions were; one cycle of 94°C (1 min), 32°C (1 min), 72°C (2 min), followed by 45 cycles 94°C (30 sec), 35°C (1 min), 72°C (1 min 30 sec), and finally, one cycle at 72°C (5 min).

Attempts were made to amplify nuclear rDNA from mitochondrial DNA preparations in order to detect if the probes were contaminated with nuclear DNA. The lower limit of detection of rDNA in a dilution series of purified nuclear DNA was found to be 320 fg - an order of magnitude less than the minimum amount of mitochondrial DNA that was found to be required

to produce a hybridization signal against target DNA blots. No rDNA was amplified from any of the mitochondrial probes purified from intact mitochondria or taken from the upper mitochondrial band found on CsCl gradients - some products were generated when lower mitochondrial bands were examined.

Restriction analysis of DNA, blotting, probing etc

RNA was removed from samples by digestion with RNAse A according to the manufacturers protocol. A subset of the DNA samples were cut with a range of restriction enzymes (BamHI, BstU1, ClaI, EcoRI, EcoRV, HaeIII, HinfI, MspI, PstI, PvuII and Sau3A) using the buffer systems and reaction conditions recommended by the manufacturers.

Final analyses were performed on DNA cut with the 6-base cutter, EcoRV in 50 mM Tris.HCl, 50 mM NaCl, 10 mM $MgCl_2$. Approximately 150 ng digested DNA was loaded per sample onto 0.7% or 1.0% agarose gels and then separated in 1 x TBE buffer for 720 volt hours at room temperature (Sambrook *et al.*, 1989). DNA fragments were transferred to Hybond N (Amersham) according to the 'Southern method', using neutralizing solution as the mobile phase.

Mitochondrial and nuclear probe DNAs (10 ng), were radiolabelled with [^{32}P] α-dCTP using the random hexamer oligo-labelling method of Feinberg & Vogelstein (1983). Prior to use, probes were denatured at 100°C for 10 min. Hybridization of both nuclear and mitochondrial DNA probes to target blots was performed under high stringency conditions; washes were performed at high stringency for mitochondrial DNA probes and at lower stringency for nuclear DNA probes (Westneat *et al.*, 1988). When appropriate, blots were re-probed following removal of 'old' probe by boiling the membranes in 0.1 x SSC, 1% SDS and then washing in cold 2 x SSC.

Competitive hybridization studies were performed by probing blots with radiolabelled t-strain mitochondrial DNA and a hundred fold excess of 'cold' non t-strain DNA. In order to determine the lower limit of probe DNA required to generate a positive signal from standardized target blots, mitochondrial DNA samples were diluted over a wide range prior to use as probes.

dsRNA detection

A search for dsRNA was performed on samples of total genomic, nuclear and mitochondrial DNA preparations following the methods of Marino *et al* (1976). This approach relies on the differential activity of RNAse A towards dsRNA compared to ssRNA under high and low salt conditions.

DNA samples, either cut with EcoRV or uncut, were precipitated with 3 M sodium acetate at -80°C for 15 min, and then washed twice with 70 % ethanol. Samples were air-dried for 45 min and then re-dissolved in sterile distilled water. All samples (300 ng DNA) were split equally into three treatments. Treatments were; (1) 100 mM sodium acetate pH 5.2, 5 mM $MgSO_4$, 40 μg ml^{-1} DNAse I for 30 min at 25°C; (2) 150 mM NaCl, 15 mM sodium citrate pH 7.4, 10 μg ml^{-1} RNAse A for 30 min at 37°C or, (3) 15 mM NaCl, 1.5 mM sodium citrate pH 7.4, 10 μg ml^{-1} RNAse A for 30 min at 37°C.

Treated DNA samples, along with digested and uncut untreated controls, were run on 0.7% agarose gels (1 x TBE) and then blotted. Treated and untreated probe DNAs were then hybridized to the blots as described previously.

Prokaryotic cytoplasmic determinants of incompatibility.

Normal homokaryons and t-strain homokaryons were grown for 4 wk on MEA plates or on plates supplemented with either 0.5 μg ml^{-1} rifampicin or 0.5 μg ml^{-1} tetracycline. Subcultures from the treated and untreated strains were then paired on MEA or MEA + antibiotic plates. Patterns of interaction and heterokaryon emergence were followed at regular intervals over 4 wk.

MtDNA haplotype analysis

Total genomic DNA samples were prepared from the seven progenitor strains and a total of 195 hyphal-tip derived strains according to the method of Raeder & Broda (1985) - as described earlier. Samples were digested with RNAse A and EcoRV. Approximately 150 ng digested DNA per sample was run for 720 volt hours at room temperature in 0.7% agarose (1 x TBE). DNA fragments were then transferred to Hybond N (Amersham) using neutralizing solution as the mobile phase.

Mitochondrial DNA probes were prepared from strains F2·109, EP1·7 and AF1·7 according to the method of Rogers, Buck & Brasier (1987) and radiolabelled with [^{32}P] α-dCTP using the random hexamer oligo-labelling method of Feinberg & Vogelstein (1983). Hybridization of probe DNA to blots and subsequent washes were performed under high stringency conditions, following the methods of Westneat *et al* (1988).

RESULTS

Somato-sexual responses in 'old-stock' sib-related strains

Pairings between original sib-related F2 fb (Table 6.3a) derived strains were consistent with a unifactorial (bipolar) homogenic system of mating-type incompatibility - reinforcing numerous past studies on mating behaviour in *Stereum hirsutum* (Ainsworth & Rayner, 1989; Coates, Rayner & Todd, 1981...) For clarity the C-factor terminology of Coates, Rayner & Todd (1981) is adopted to describe the mating-types of strains. Of the 27 isolates examined, 13 possessed the C1 allele and 14 the C2 allele; suggesting no significant departure from a random pattern of segregation.

Incompatible (homoallelic) pairings were characterized by the formation of degenerative lysed demarcation zones between the interactants, accompanied by the production of ochraceous to umber pigmentation (Figure 6.1). The intensity of somatic incompatibility responses varied. Some isolates consistently produced strong somatic incompatibility responses, whereas in the majority of pairings, the responses were both more variable and weaker. For some pairings, qualitative changes in the morphology of the interacting mycelia were not detected. Such interactions were often accompanied by the build up of an aerial barrage along the interfacial zone.

Most incompatible pairings resulted in the formation of 'bow-tie' like zones which were either unilateral or bilateral in extent (Coates *et al.*, 1981; Coates & Rayner, 1985b). Moreover, 'bow-tie' zones were produced more frequently than would be expected from past studies of incompatible pairings (Coates & Rayner, 1981).

No permanent somatic incompatibility was evident in compatible matings (Figure 6.1). Colonies merged to produce uniform mycelial mats that differed morphologically from both of the progenitor strains. Upon subculturing, heterokaryons produced speckled, ropey mycelia accompanied by the generation of clear colourless to luteus watery droplets. Some compatible pairings produced weak 'bow-tie' like zones that eventually occupied the entire area of the interaction plates. In contrast to the incompatible pairings, fewer 'bow-tie' interactions were observed than expected.

Table 6.3. *Mating type interaction outcomes of (a) 'old' stock culture strains and, (b) repeatedly sub-cultured working strains of Stereum hirsutum F2-series progeny.*

(a)

| | | |

Figure 6.1. Outcome of pairings between homokaryotic *Stereum hirsutum* F2 progeny series strains; (a) normal incompatible and compatible mating-type responses, (b) incompatible homo-allelic mating responses amongst working strains of the C1 mating-type - below the diagonal, and amongst the C2 mating-type - above the diagonal. Figure continued overleaf.

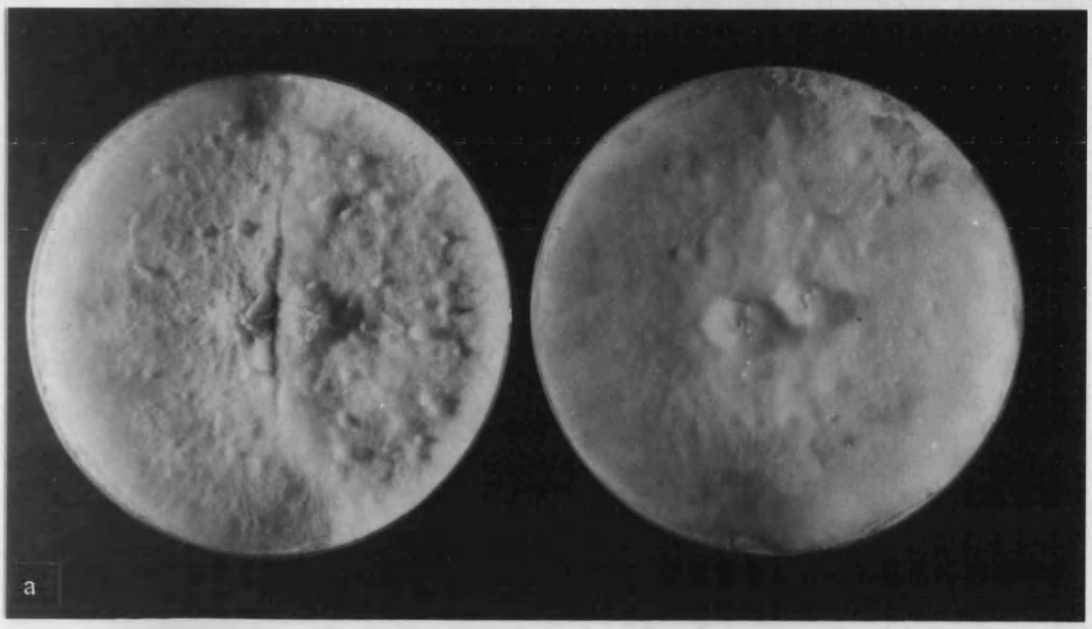
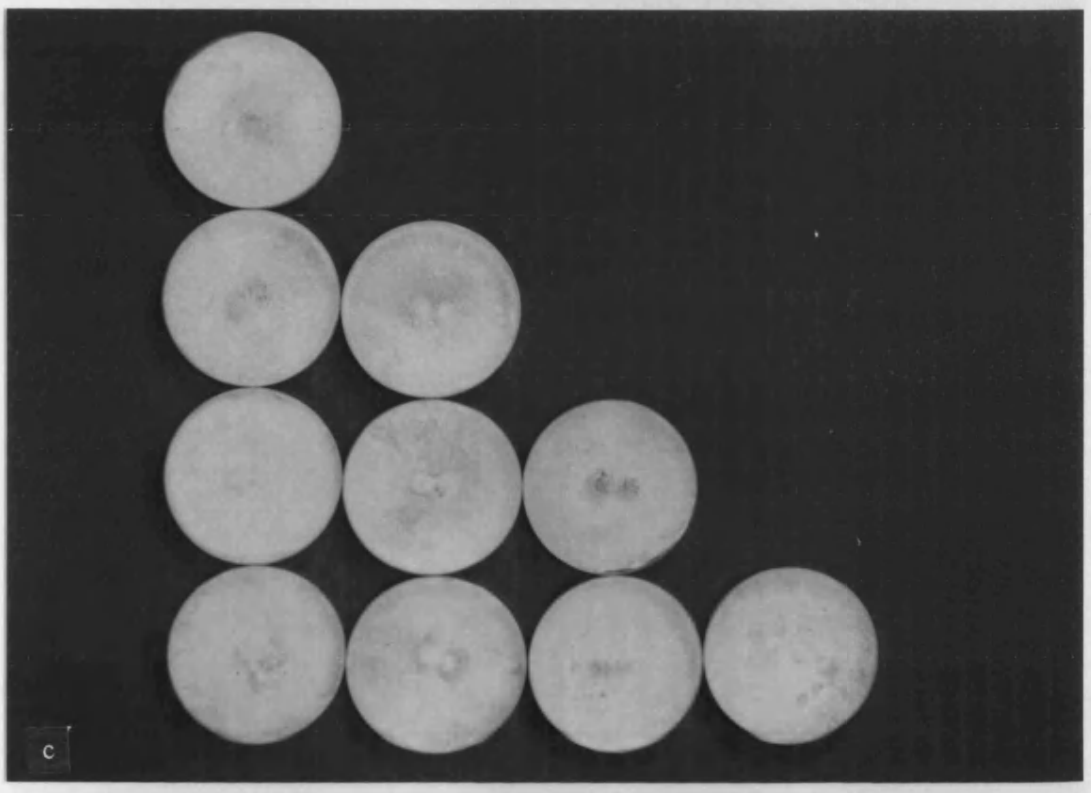
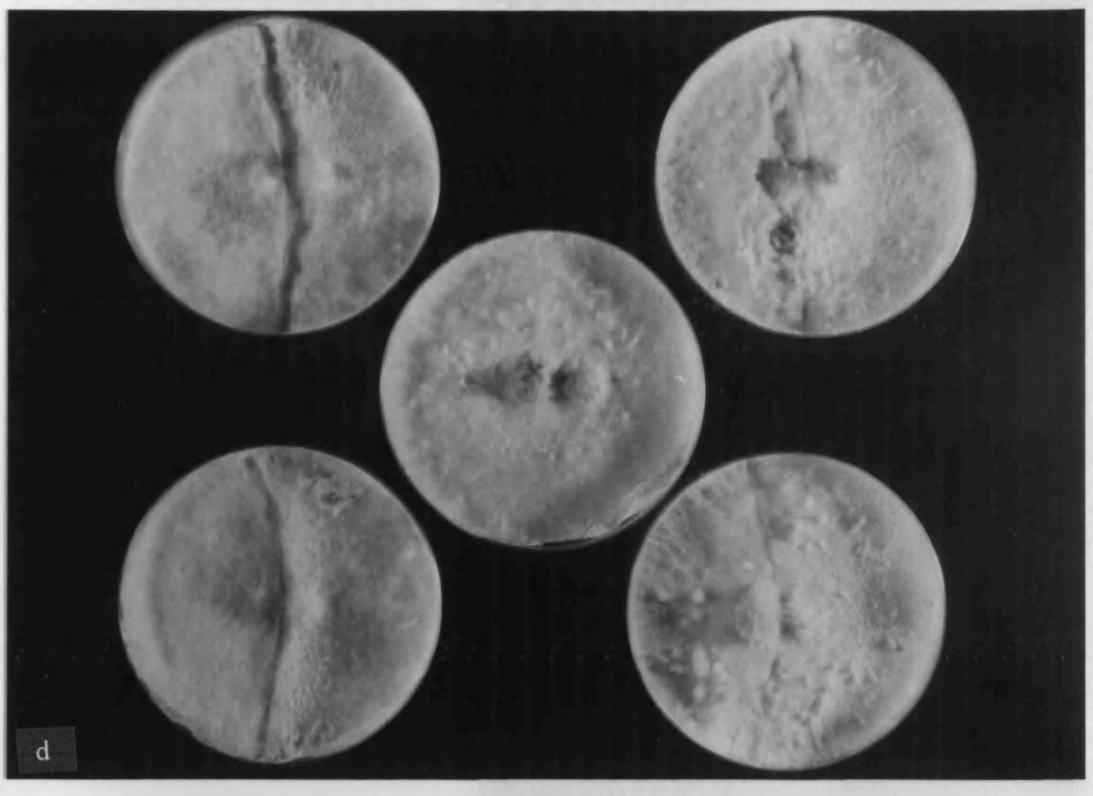


Figure 6.1 continued. Outcome of pairings between homokaryotic *Stereum hirsutum* F2 progeny series strains; (c) aberrant fully compatible matings between working strain F2-sibs with the same mating-type, (d) normal incompatible matings between 'old-stock' F2.116 and working strain F2-sibs of the C2 mating-type group.





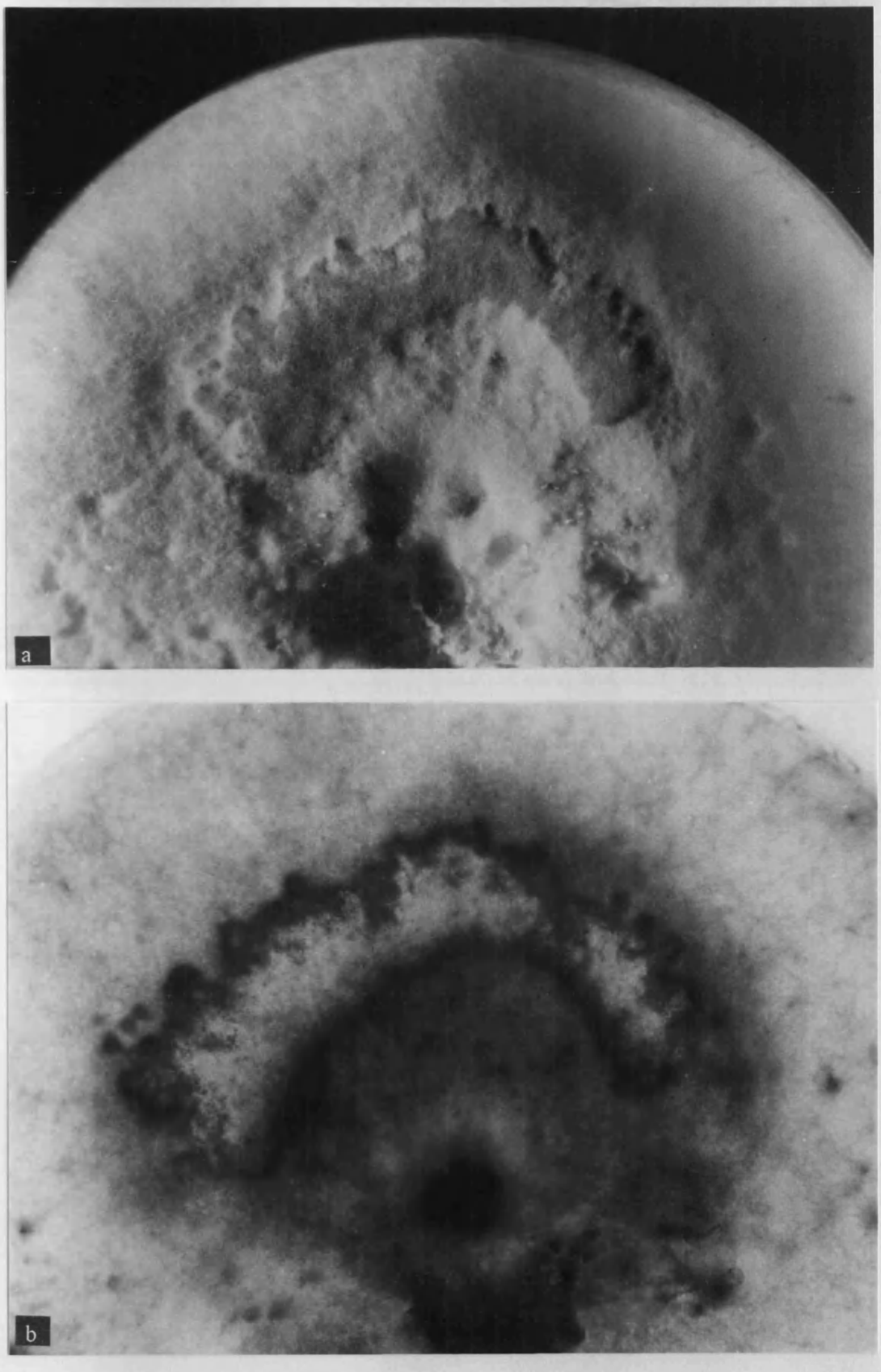
An unusual outcome was observed in 13 out of 196 fully compatible pairings, manifested by the formation of a 'crescent-shaped' degenerative zone behind the inoculum plug of one of the interactants (Figure 6.2). This zone lacked significant aerial growth, produced an olivaceous to ochraceous mycelium speckled with luteus pigment and was bounded on the outermost edge by luteus droplets. In all cases, mycelium distal to the inoculum plug eventually recovered to produce normal heterokaryotic growth. The expression of a 'crescent' was always unilateral and apparently sporadic in occurrence. Crescent zones were induced in 7 out of the 27 strains examined, and were mostly observed in strains with the C1 mating-type (11 / 13 cases). Strains F2.4 and F2.101 exhibited the crescent phenotype on four and three occasions respectively. For the remaining strains the phenotype was only observed in a single pairing; no single strain consistently induced the crescent. Crescents were produced by strains derived from both natural and laboratory-induced fruitbodies.

Altered somato-sexual responses in 'sub-cultured' homokaryotic sib-related strains

Isolates sub-cultured many times were phenotypically indistinguishable from strains of equivalent genotype that had been maintained as stock-cultures. The majority of the 27 strains examined conformed to the mating-type interaction patterns expected, but no 'bow-ties' were observed in any of the incompatible pairings. Moreover, one strain with the C1 mating-type allele, and three strains with the C2 mating-type allele failed to show signs of somatic incompatibility when paired with any of the other members of the F2-series progeny set, irrespective of their mating-type (Table 6.3b). All pairings involving these 'altered' strains resulted in the bilateral formation of a ropey or crust-like, speckled mycelium with small luteus watery droplets over the surface (Figure 6.1c). Phenotypically these resembled the heterokaryons derived from normal fully compatible matings. Pairings between the four 'altered' strains themselves also resembled fully compatible matings.

Pairings between normal strains and the parental fruitbody isolate, F2 fb, or the 'transformed' strain, F2.4t, resulted in the expression of a strong somatic incompatibility response. When 'altered' strains were paired against F2 fb only a weak rejection response was observed; a slightly stronger response was elicited against F2.4t.

Figure 6.2. A crescent shaped lytic zone produced behind the inoculum plug of *Stereum hirsutum* strain F2.4 when paired against F2.102; (a) surface view, (b) reverse.



Outcome of pairings between homokaryotic and heterokaryotic strains

Pairings between the non-sib related homokaryons were all fully compatible (Figure 6.3b,c,j,k). A weak somatic incompatibility response was evident initially but no significant cell death occurred. Single-hyphal tip cultures from these pairings produced uniform mycelia with a heterokaryotic phenotype. Some tip-derived strains from the F2.101 side of the EP1.7 x F2.101 pairing produced colonies with sparse, submerged hyphae, giving the mycelium a water soaked appearance (Figure 6.4).

Homokaryon and heterokaryon pairings were more variable (Figure 6.3a,d,f,g,i,l). When AF1.6 or EP1.7 was paired against F2 fb or F2.4t, the heterokaryons remained largely unaffected by the presence of the homokaryon. No variation was detected amongst hyphal-tip progeny obtained from the heterokaryotic parental side of the interactions. In contrast, the homokaryons produced an extensive somatic incompatibility response along the interfacial boundary prior to the conversion of the homokaryon into a speckled heterokaryotic phenotype. Hyphal-tip progeny taken from this side of the interaction were again mostly uniform in appearance, though some degenerative phenotypes were produced that resolved into sectors with various parental and non-parental phenotypes. In the EP1.7 x F2 fb pairings these degenerate colonies were restricted to the interior of the domain originally occupied by the EP1.7 progenitor. Colonies derived from the appressed EP1.7 margin of the interaction plates showed no degeneracy. This contrasted with the outcome of the pairing of EP1.7 and F2.4t, where degenerate phenotypes were only recovered from the immediate interaction zone. The intensity of the initial somatic incompatibility response was weaker against the more closely related AF1.6 isolate than against EP1.7. Morphological changes in the homokaryotic mycelium were most rapid when AF1.6 was the acceptor mycelium.

Interaction outcomes between the F2 homokaryons and the EP fb heterokaryon were very different and more complex. As before, the heterokaryon remained largely unchanged, though portions of the mycelium did possess a non-parental, pale luteus, powdery or farinaceous appearance. Hyphal-tip subcultures taken from the EP fb side of an interaction resembled the parental heterokaryon, but were more prone to producing internal pseudosclerotial plates or barriers (Figure 6.4c). On the homokaryotic side of the interactions, a wide degenerate

Figure 6.3. Pairings set up to examine mitochondrial DNA transmission patterns between strains of *Stereum hirsutum*; (a) AF1.6 x F2 fb, (b) AF1.6 x F2.10, (c) AF1.6 x F2.101, (d) AF1.6 x F2.4t, (e) EPfb x F2 fb, (f) EPfb x F2.10, (g) EPfb x F2.101, (h) EPfb x F2.4t, (i) EP1.7 x F2 fb, (j) EP1.7 x F2.10, (k) EP1.7 x F2.101, (l) EP1.7 x F2.4t.

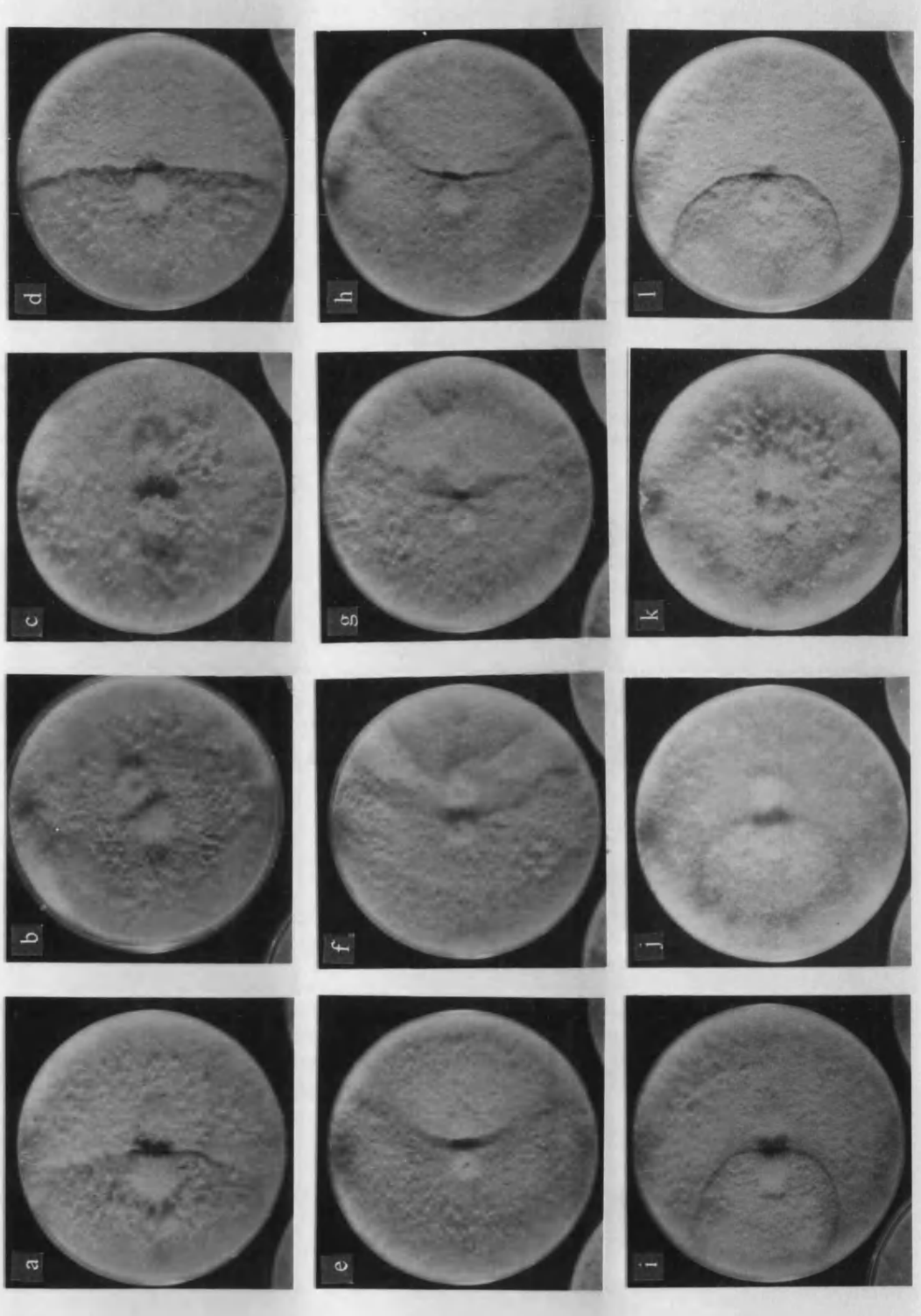
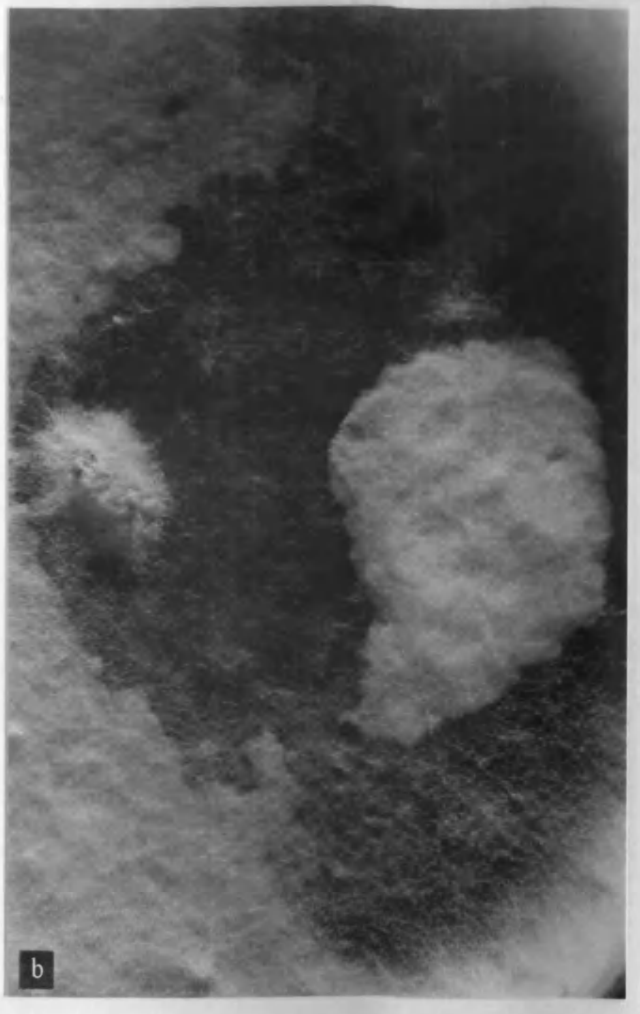
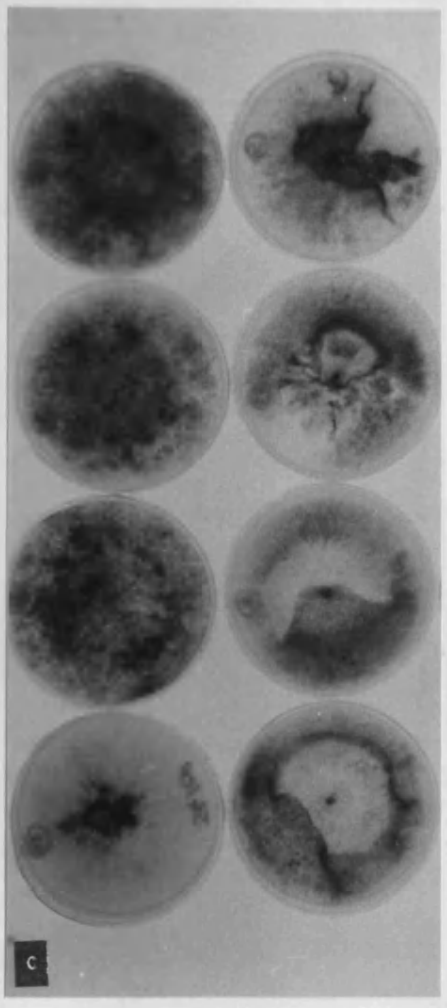


Figure 6.4. Variation in hyphal-tip progeny isolated from the pairings set up to examine patterns of mitochondrial transmission; (a) Sectoring of hyphal-tip derived strain to produce a pigmented degenerate zone, bounded by watery droplets, (b) appressed mycelium with a grazed 'wet' appearance produced by hyphal-tip strains from the F2.101 side of an interaction between EP1.7 and F2.101, (c) reverse view of a range of single hyphal-tip derived colonies exhibiting a variety of types of degenerate growth and switches.





appression zone developed. This region extended along the entire length of the mycelial interface, and then continued in towards the centre of the domain originally occupied by the homokaryotic progenitor. Hyphal-tip progeny from this zone were very variable, and were mostly degenerate. After 4 wk, the margins of the colonies still contained mycelia that resembled the homokaryotic parent, but on sub-culturing both homokaryotic and variable non-parental colony types were recovered.

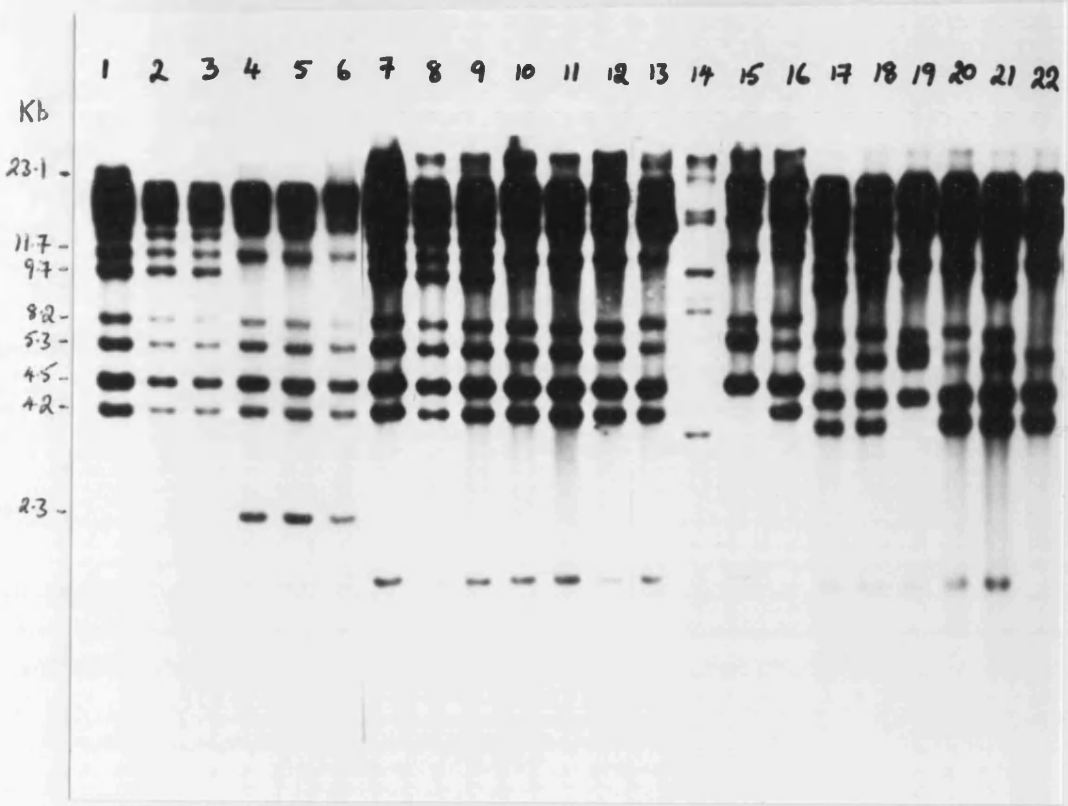
Interactions between heterokaryons resulted in the expression of strong somatic incompatibility responses which were restricted to a narrow zone (<3mm wide) between the mycelia (Figure 6.3e,h). No changes in the morphology of the interactants themselves were detected. Hyphal-tips prepared from either side of an interaction produced colonies identical to their progenitor strains. However, several unusual colony types, in addition to degenerate forms, which were incompatible with either of the parental strains were recovered from the interaction interface itself.

Mitochondrial DNA variation between populations

EcoRV digested total genomic DNA samples of *Stereum hirsutum* obtained from diverse geographical locations were probed with purified mtDNA. Patterns of hybridization revealed a high degree of variability between strains (Figure 6.5). MtDNA molecules ranged in size from 69.5 kb for the *Stereum complicatum* strain to 99.9 kb for *S.hirsutum* strain AF1.6. Within the British isolates, mtDNA sizes varied from 89.0 to 99.9 kb (mean 93.9 ± 1.9 kb). Much of this variation was apparent as length alterations amongst common core sequences that did not introduce novel EcoRV sites.

A comparison of hybridization patterns indicates that both F2 and U11 possessed unique sequences; F2 contained a 2.3 kb fragment and U11 a 3.3 kb fragment. Strains AF1.6 and EP18.7 produced a 4.1 kb fragment which was not possessed by any other strains and which had no homology to other mitochondrial DNAs. Most mtDNAs contained a 4.2, or 4.5 kb fragment, many contained both. These fragments were partially homologous, since possession of either one produced a positive signal in strains that contained both. Mitochondrial DNA from strain PB3.14 lacked any homology to either of these fragments.

Figure 6.5. Mitochondrial DNA variation in strains of *Stereum hirsutum*. Lane (1) EP1.10, (2) EP1.12, (3) EP1.13, (4) F2.101, (5) F2.107, (6) F2.110, (7) EP1.10, (8) EP1.12, (9) EP1.13, (10) F2.101, (11) F2.107, (12) F2.110, (13) BR1, (14) JG1.5, (15) HH1, (16) U11, (17) EP1fb, (18) G2, (19) NF1, (20) AF1.6, (21) EP1.7 and, (22) EP18.7. Probes used were F2.103 mt (lanes 1-6), BR2 mt (lanes 7-12), EP1.5 mt (lanes 13-16) and F2.9 (lanes 17-22).



Differential hybridization

When total genomic DNA samples were probed with mtDNA isolated from strain F2.4t, positive signals were obtained from fragments that were not located within the mitochondrial genome - see Figure 6.6. These included a large fragment, greater in size than the 23.6 kb fragment of HindIII cut lambda, and a variable number of small fragments (> 1 kb). MtDNA probes isolated from sib-related, non-'transformed' homokaryons F2.4, F2.9 and F2.10 did not pick out any of these bands even when the same blots were re-probed. MtDNA from the parental heterokaryotic strain (F2 fb) produced the same pattern as F2.4t.

The additional high molecular weight band was present in all of the 41 *Stereum* strains examined. The possibility that this band was uncut mtDNA was eliminated, since blots could give a positive signal with one probe, and then after stripping, no signal with another related probe. The low molecular weight fragments were only present in F2 and EP1 strains and length polymorphisms could only be detected in the F2 series progeny strains.

When mtDNA samples were prepared from 32 different source strains and used to probe standardized blots, probes derived from eight heterokaryotic mycelia, two 'transformed strains' and five homokaryons, picked out the additional bands. Of the remaining 17 strains (all homokaryotic), 11 did not produce any signal from the additional fragments, whilst six produced no signal in an initial screening but picked out the bands in a later investigation (Table 6.4).

CsCl gradients

Genomic DNA samples from a range of homokaryotic and heterokaryotic strains were fractionated using bis-benzimide CsCl buoyant density ultracentrifugation. Bands visible under short wave UV were located at three positions (Figure 6.7). The dominant bottom band, (ρ = 1.68 - 1.60 g cm^{-3}) contained nuclear DNA (confirmed by DNA fingerprinting with Jeffreys' probe, 33.15). Restriction analysis and hybridization studies revealed that the two additional bands (ρ = 1.60 and ρ = 1.58 g cm^{-3}) contained mtDNA contaminated with small amounts of nuclear DNA and pure mtDNA respectively. Most mtDNA was located in the middle band. The low density band was detected in strains F2.4t, F2.4rnt, F2.9, F2.9t, F2.9rnt, F2.10, G2 fb and NF1 fb. It could not be detected in F2.4, F2,4rt, F2 fb or EP fb.

Figure 6.6. Mitochondrial DNA samples from sib-related homokaryotic strains F2.9 and F2.4t give different hybridization patterns when used as probes against total genomic (tg) DNA EcoRV digests, but identical results against purified mitochondrial (mt) DNA digests. (a) F2.9 mt DNA used as probe against target DNA samples, (1) F2.4 tg; (2) F2.4 mt; (3) F2.9 tg; (4) F2.9 mt; (5) AF1.6 tg; and (6) AF1.6 mt. (b) F2.4t mt DNA used as probe against target DNA samples, (1) F2.4 tg; (2) F2.4 mt; (3) F2.9 tg; (4) F2.9 mt; (5) AF1.6 tg; and (6) AF1.6 mt.

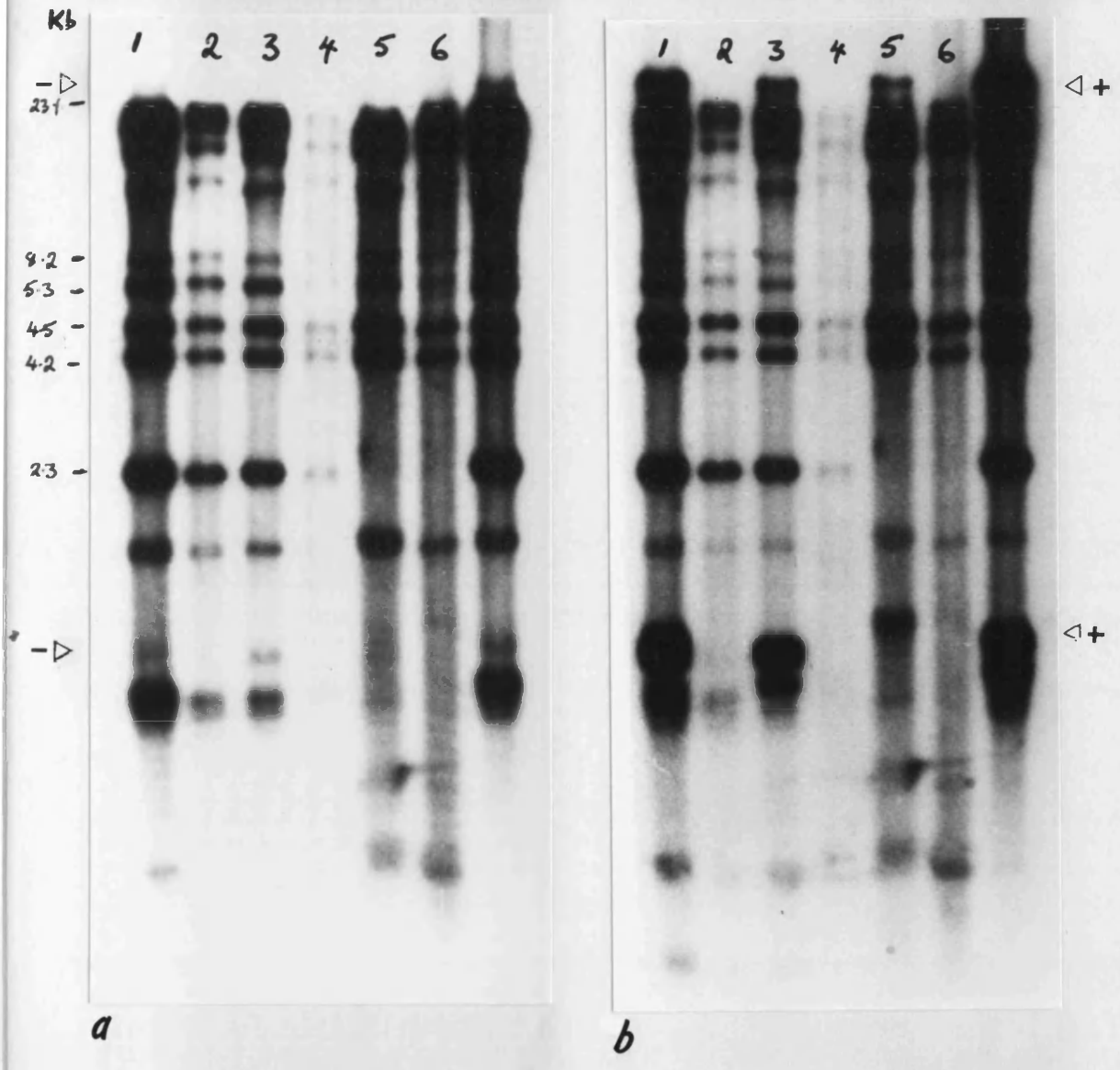
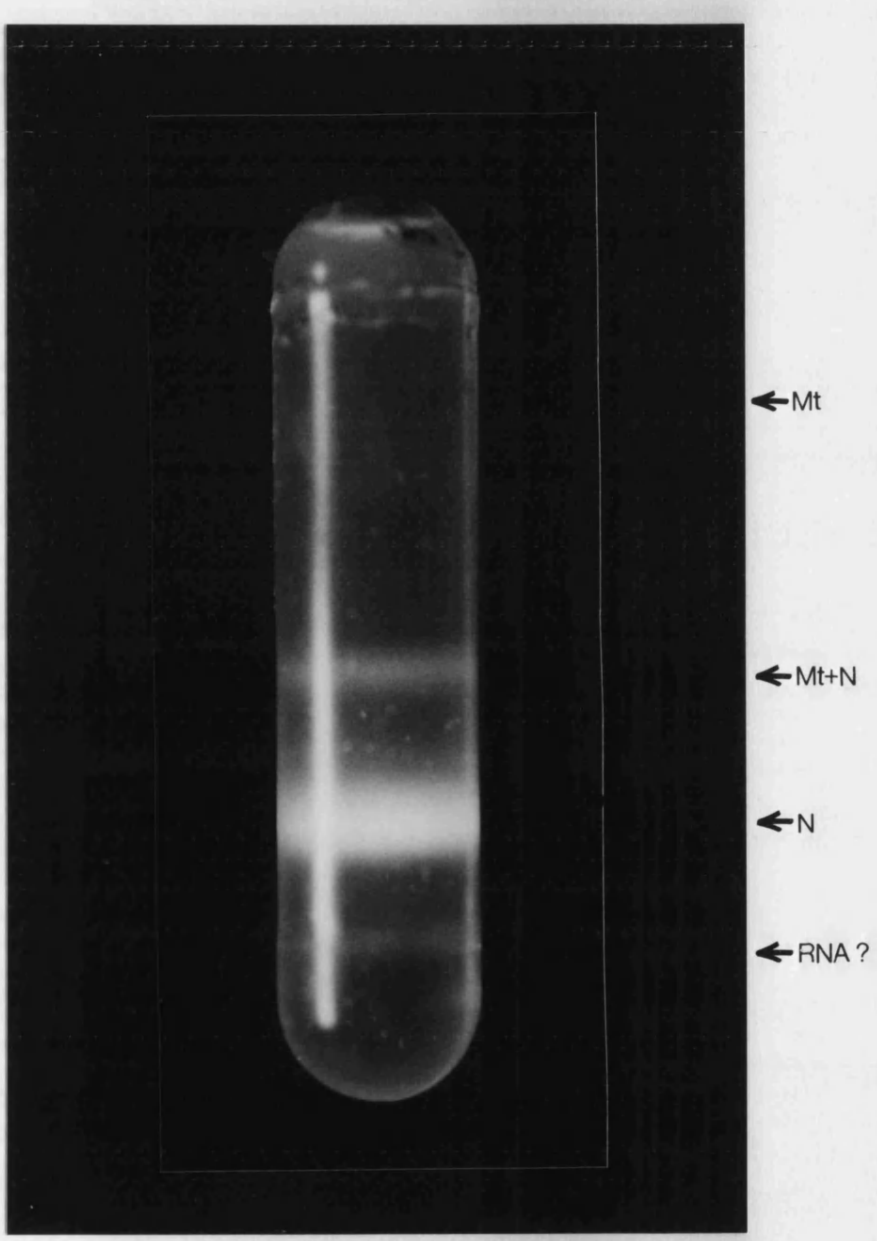


Table 6.4. *Mitochondrial DNAs from Stereum hirsutum homokaryotic and heterokaryotic source strains found to detect a high molecular weight band in EcoRV digested total genomic DNAs.*

Signal (+)	**Signal (+)**	**Signal (-)**	**Signal (+/-)**
Heterokaryons	Homokaryons	Homokaryons	Homokaryons
F2 fb	F2·107	F2·9	F2·4
EP1 fb	F2·110	F2·10	F2·115
BR1 fb	F2·118	F2·101	F2·117
BR2 fb	EP1·3	F2·102	EP1·2
G2 fb	PB3·14	F2·103	EP1·4
NF1 fb	EP1·6	EP1·5	
HH1 fb	Transformed	EP1·7	
U11 fb	homokaryons	EP1·9	
		EP1·11	
	F2·4t	EP18·7	
	F2·9t	AF1·6	

Figure 6.7. CsCl/bis-benzimide gradient separation of total genomic DNA from *Stereum hirsutum* strains F2.4t, F2.4rnt and F2.9t gives four well separated bands. An upper mitochondrial DNA band was not apparent in strains F2.4, F2.4rt, F2.9, F2.9rnt and F2.10.



By probing EcoRV digested CsCl purified nuclear and mtDNA samples with pure mitochondrial DNA it became clear that the high and low molecular weight fragments detected by certain mtDNA probes were located in the nuclear fraction (Figure 6.8, 6.9, 6.10a). When blots supporting EcoRV digested total genomic DNA were probed with purified nuclear DNA, the strongest signals were also obtained from positions equivalent to those of the additional fragments. These observations suggest that the fragments picked out by mitochondrial probes contain highly repetitive sequences that co-purify with nuclear DNA. Radiolabelled probe nuclear DNA did not give any signal against blots with purified mtDNA target samples. Even though variation was apparent in the target nuclear sequences, nuclear DNA samples prepared from different sources all gave the same hybridization patterns.

To assess the likelihood that pure mtDNA was contaminated with nuclear DNA, a number of analyses were performed. Firstly, using the rDNA PCR primers of Sogin (1990) and White *et al* (1990) single pure 2 kb products were generated from both purified nuclear and total genomic DNA samples. When purified nuclear DNA was included in amplification reactions at concentrations ranging from 3.0 ng μl^{-1} to 0.3 pg μl^{-1}, products were obtained from all of the reactions. No product was obtained from the control tube lacking DNA (Figure 6.11a). No products were amplified from any of the probe mtDNA samples examined, therefore if nuclear rDNA was present in the probe samples, there must have been less than 0.3 pg μl^{-1} (Figure 6.11b). Such a low level of 'contamination' would have been insufficient to give a positive hybridization signal when against target DNA samples since a minimum of 100 pg of mtDNA was required to give a signal against standardized blots supporting 250 ng target DNA - Figure 6.12.

Other observations

MtDNA samples prepared from sib-related homokaryons and their progenitor parent heterokaryons showed no differences when they were cut with twelve different restriction enzymes (Figure 6.13). The low molecular weight fragments picked out by mtDNA were only generated when nuclear DNA samples were cut with EcoRV. Fragments of nuclear rDNA (2kb), purified from PCR amplifications, hybridized strongly to the top high molecular weight band, but gave no signal at the lower molecular weight positions (Figure 6.10b). Treatment of

Figure 6.8. EcoRV cut DNA samples from different sources probed with; (a) F2 fb nuclear DNA and (b) F2.9t mtDNA. Lanes contain (1) F2.4t total genomic DNA, (2) F2.9 total genomic DNA, (3) F2.4t CsCl purified nuclear DNA, (4) F2.9 CsCl purified nuclear DNA, (5) F2.4t mtDNA, (6) F2.9t mtDNA, (7) F2.4t CsCl purified high density mtDNA, (8) F2.9t CsCl purified high density mtDNA, (9) F2.4t CsCl purified low density mt DNA and (10) F2.9t CsCl purified low density mtDNA. Note that the positive hybridization signals from the nuclear DNA correspond to those picked out by mtDNAs from 'transformed' strains.

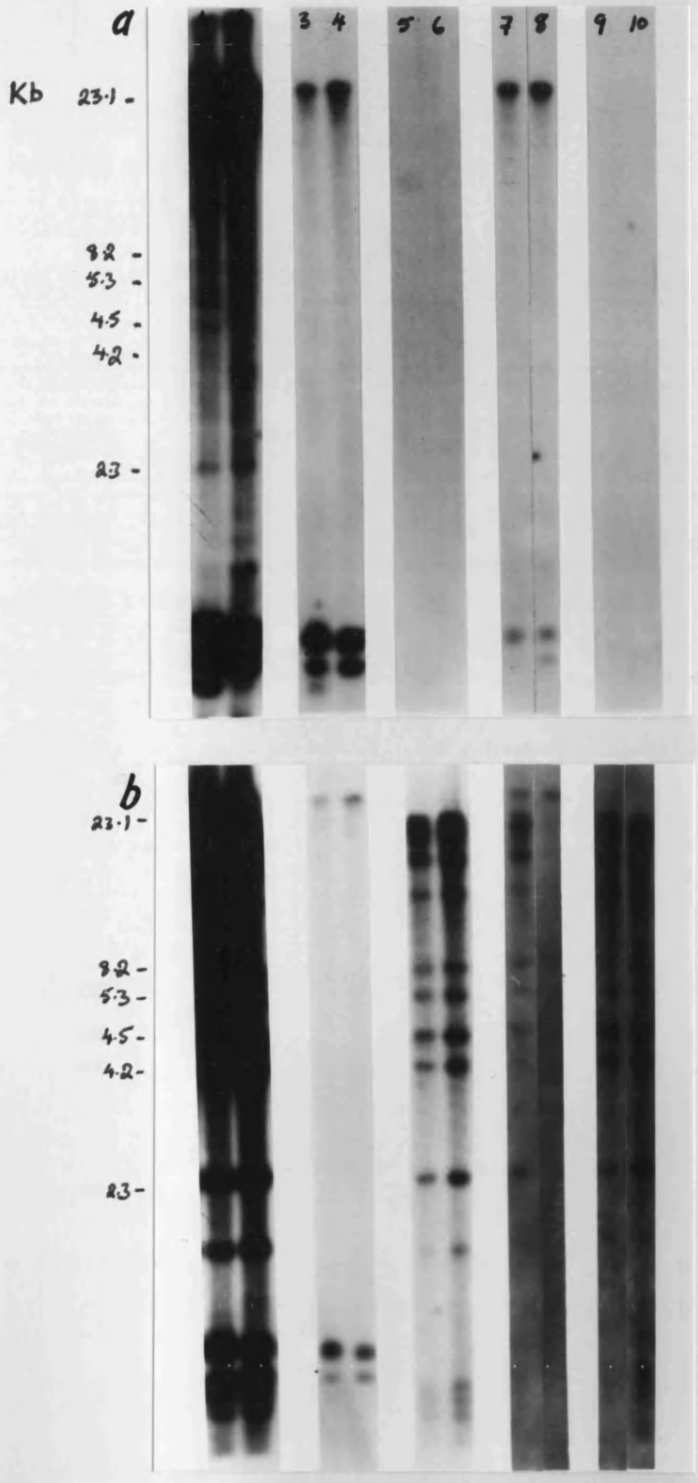


Figure 6.9. Various EcoRV cut total genomic DNA samples probed with; (a) F2.4 nuclear DNA and (b) F2.117 mtDNA. Lanes, (1) EP1.11, (2) EP1.7, (3) EP1.9, (4) EP1.10, (5) EP1.12, (6) EP1.13, (7) F2.101, (8) F2.107, (9) F2.110, (10) F2.115, (11) F2.116, (12) F2.117, (13) F2.118, (14) BR1, (15) JG1.5, (16) HH1, (17) U11, (18) F2.4t, (19) F2.4 x F2.10 heterokaryon with F2.10 mtDNA, and (20) F2.4t x F2.10 with F2.10 mtDNA, (1a) EP1.4.

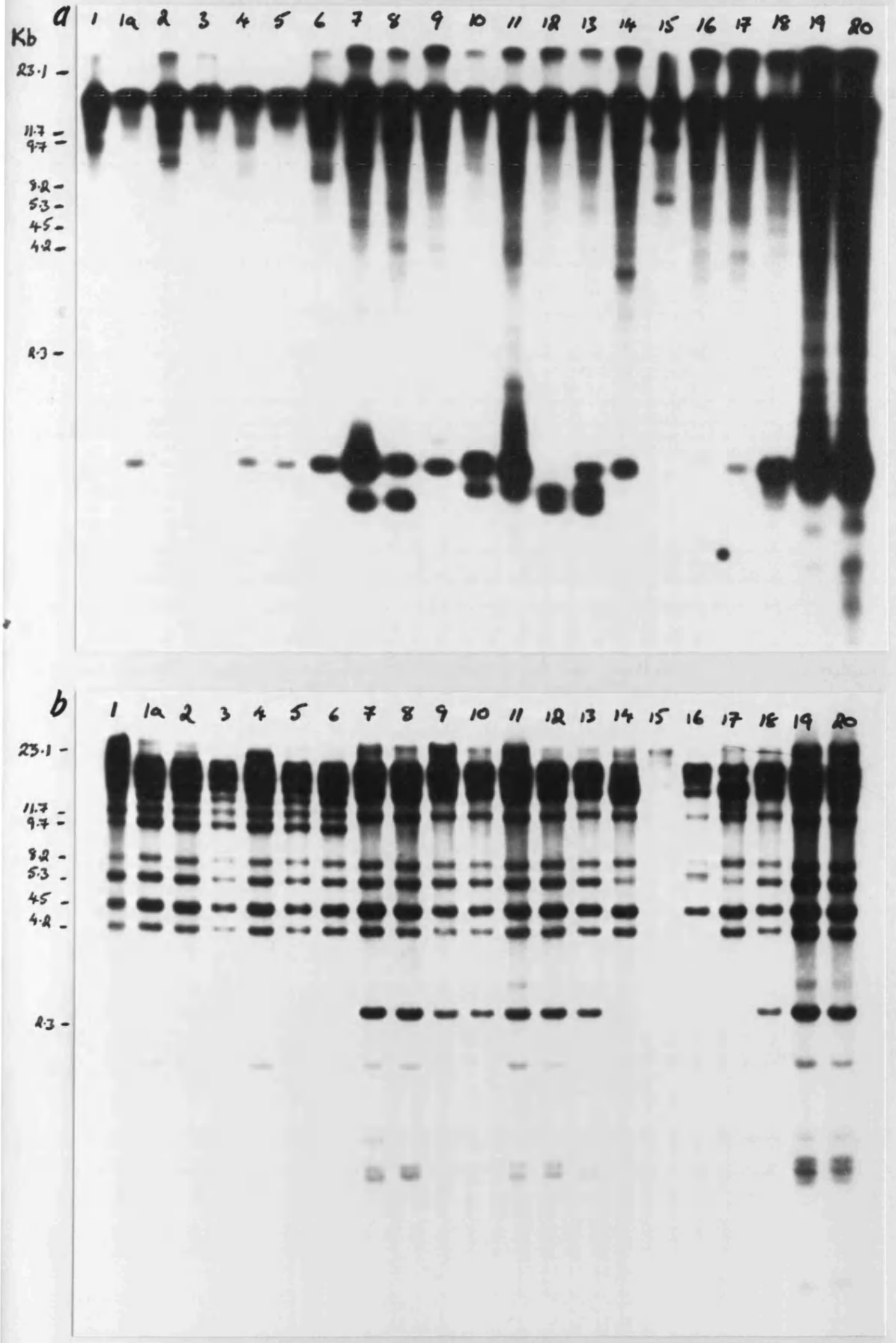


Figure 6.10. (a) F2.4 CsCl purified nuclear DNA used as probe against: lanes, (1) EP1.11, (2) EP1.7, (3) EP1.9, (4) EP1.10, (5) EP1.12, (6) EP1.13, (7) F2.101, (8) F2.107, (9) F2.110, (10) F2.115, (11) F2.116, (12) F2.117, (13) F2.118, (14) BR1, (15) JG1.5, (16) HH1, (17) U11, (18) F2.4t, (19) F2.4 x F2.10 heterokaryon with F2.10 mtDNA, and (20) F2.4t x F2.10 with F2.10 mtDNA. (b) PCR amplified 16S rDNA used as probe against: lanes, (1) EP1.11, (2) EP1.7, (3) EP1.9, (4) EP1.10, (5) EP1.12, (6) EP1.13, (7) F2.101, (8) F2.107, (9) F2.110, (10) F2.115, (11) F2.116, (12) F2.117, (13) F2.118, (14) BR1, (15) JG1.5, (16) HH1, (17) U11, (18) F2.4t, (19) F2.4 x F2.10 heterokaryon with F2.10 mtDNA, and (20) F2.4t x F2.10 with F2.10 mtDNA, (1a) EP1.4.

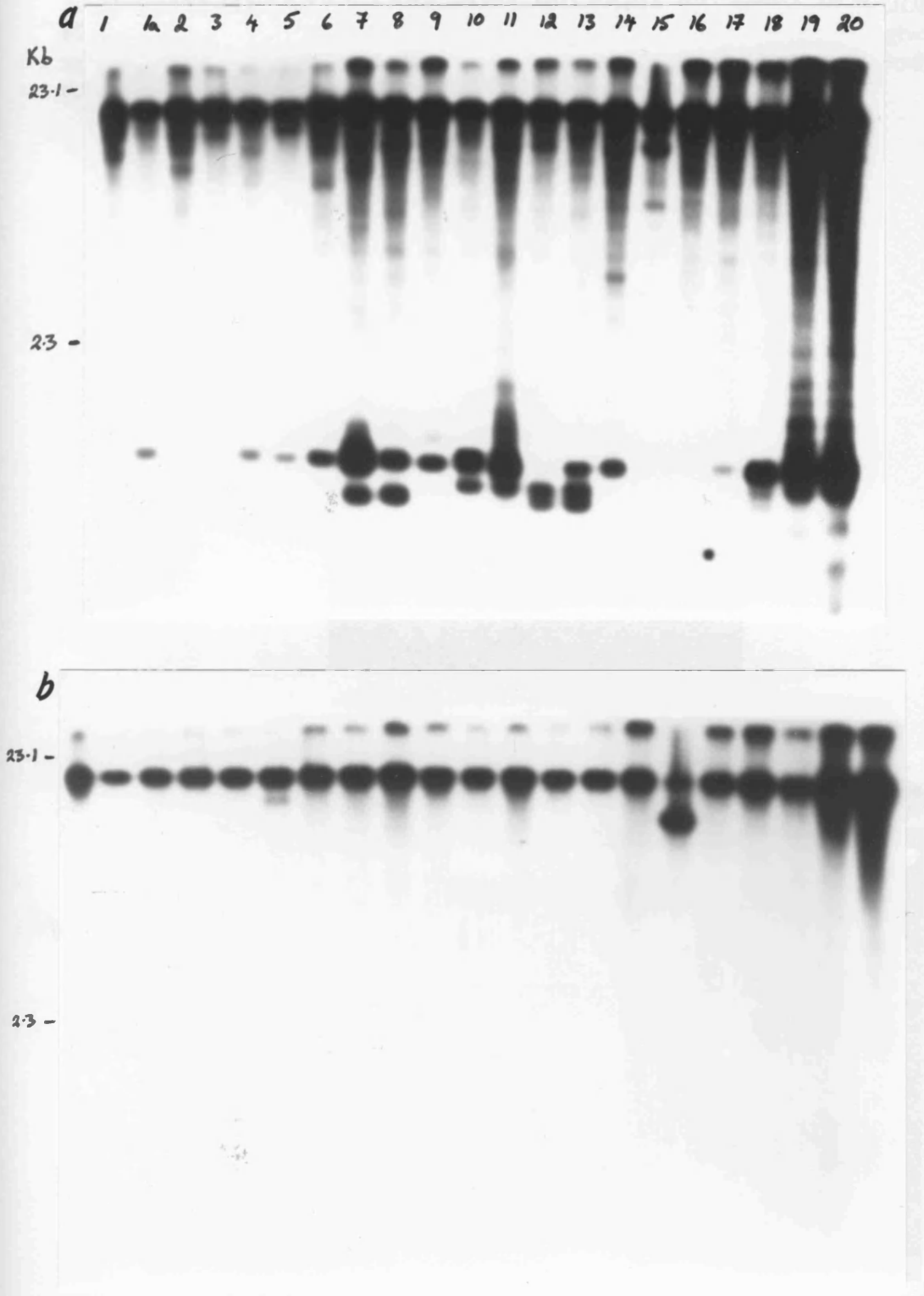


Figure 6.11. (a) 16S rDNA amplified from CsCl purified NF1 nuclear DNA with template DNA samples ranging in concentration; lanes (1) 3.2 ng μl^{-1}, (2) 1.6 ng μl^{-1}, (3) 640 pg μl^{-1}, (4) 320 pg μl^{-1}, (5) 64 pg μl^{-1}, (6) 32 pg μl^{-1}, (7) 6.4 pg μl^{-1}, (8) 3.2 pg μl^{-1}, (9) 640 fg μl^{-1}, (10) 320 fg μl^{-1}, (11) negative control, (12) 250 ng HindIII cut lambda DNA size marker. (b) Sample DNAs (1.6 ng μl^{-1}) used as template for amplification of 16S rDNA, (1) EP 1.3 mtDNA, (2) EP1.5 mtDNA, (3) U11 mtDNA, (4) H/C mtDNA, (5) JG1.5 mtDNA, (6) EP18.7 mtDNA, (7) NF1 mtDNA low density CsCl band, (8) NF1 mtDNA high density CsCl band, (9) NF1 CsCl nuclear DNA band. No amplification was evident for any of the mitochondrial DNA samples that had been used as standard probes in hybridization studies. Additional probe samples examined but not shown (CF1 mtDNA, HH1 mtDNA, BR1 mtDNA, F2.4t mtDNA, F2.108 mtDNA, F2.107 mtDNA, F2.103 mtDNA, and EP1.6 mtDNA) also failed to give any amplification products. Each pair of tracks represents the use of alternative primers, firstly NS1 - NS8 primers, and secondly A - B primers.

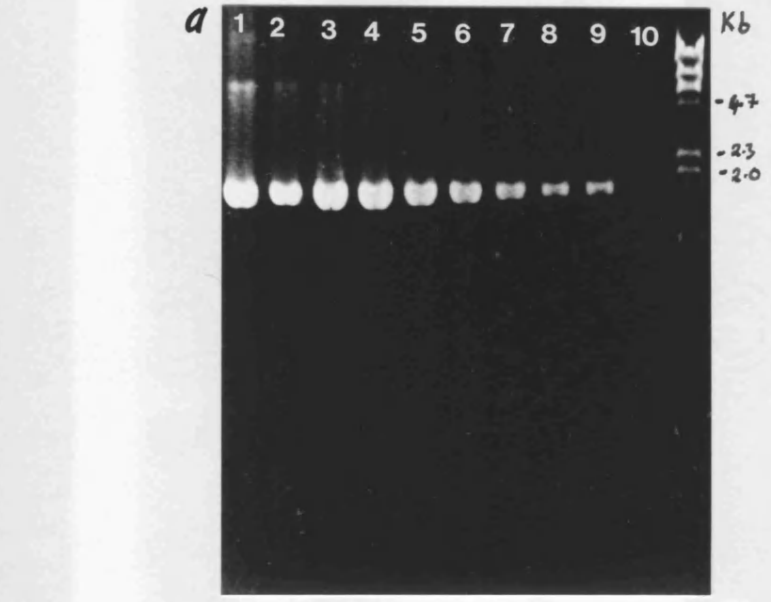


Figure 6.12. Hybridization signals of 250 ng EcoRV cut (1) F2.9 and (2) F2.102 total genomic DNA when probed with EP1.3 mtDNA at various concentrations in 3 ml hybridization solution. Lanes (a) 10 ng, (b) 5 ng, (c) 1 ng, (d) 500 pg, (e) 100 pg, (f) 1 pg, and (g) 100 fg.

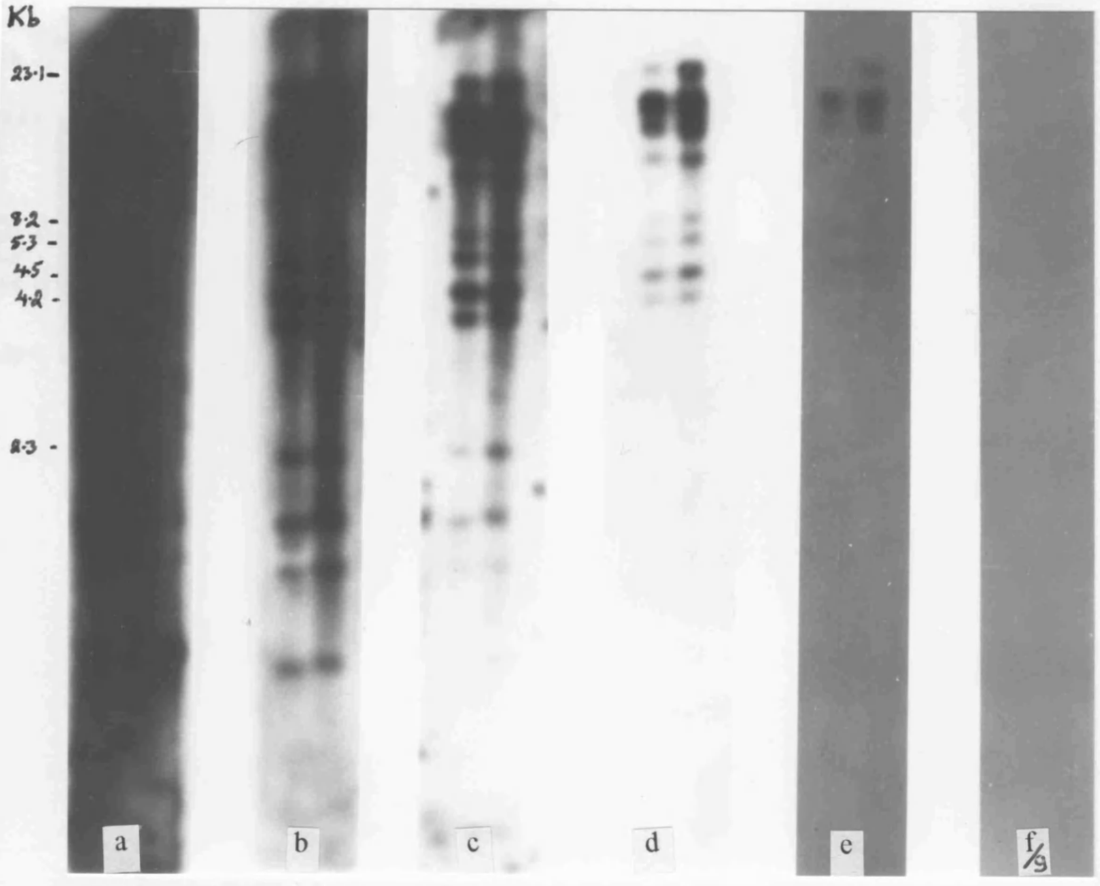
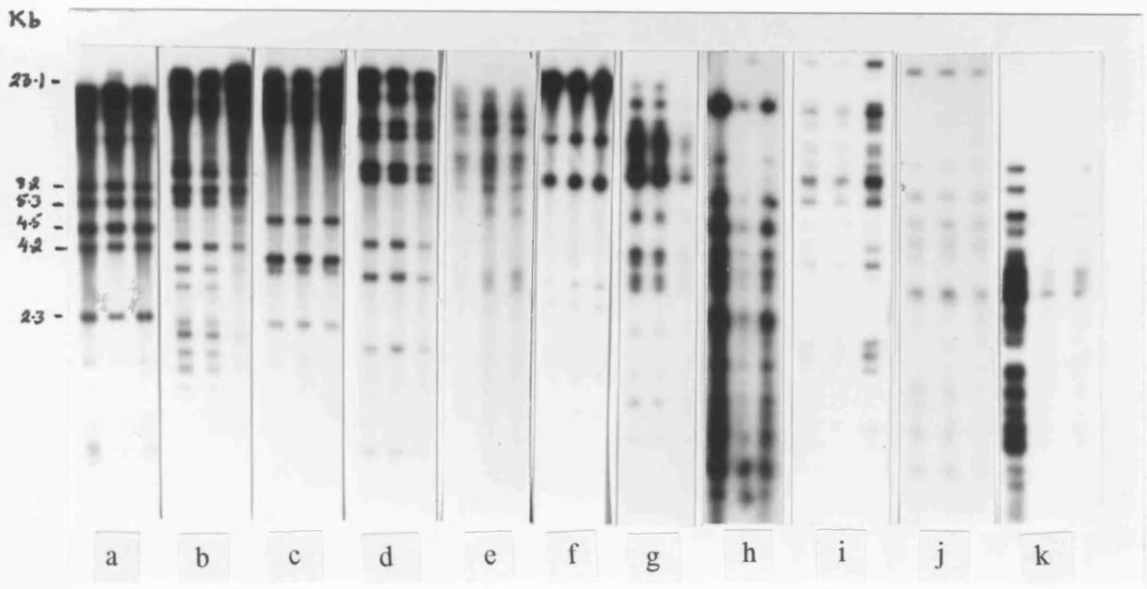


Figure 6.13. Restriction digests of total genomic DNA probed with F2.4t mt DNA. Lanes contain (1) F2.4t, (2) F2.9 and (3) F2 fb total genomic DNA. Restriction enzymes used were (a) EcoRV, (b) EcoRI, (c) BamHI, (d) PstI, (e) PvuII, (f) ClaI, (g) BstU1, (h) Sau3A,(i) HaeIII, (j) MspI, and (k) HinfI.



probe and target DNA samples with RNAse, which shows differential activity towards dsRNA compared to ssRNA under high and low salt conditions, produced no changes in either target or probe hybridization signals - whereas treatment with DNAse eliminated all signals (Figure 6.14).

Rifampicin and tetracycline were unable to 'cure' the 'transformed' strains of their heterokaryotic phenotype (Figure 6.15). The unusual somato-sexual recognition response of these strains is unlikely to be due to the presence of cryptic prokaryotic determinants of incompatibility.

Molecular analysis of F2 strains with normal and altered mating responses

When EcoRV digested total genomic DNA samples obtained from the original and sub-cultured strains were probed with mitochondrial DNA (from any of the F2-series strains), an additional 2.4 kb fragment was found in those strains with the newly described altered mating responses (Figure 6.16d).

Blots probed with DNA purified directly from the 2.3 kb band (unique to the F2 strain mitochondria) produced a strong signal against itself, and a weaker signal against the novel 2.4 kb fragment. Other fragments from the mitochondrial genome failed to hybridize to either of these bands (Figure 6.17). Mitochondrial DNA from non-F2 strains also failed to hybridize to either of these bands.

Transmission patterns of mitochondrial DNA

To facilitate easy identification and tracking of mitochondria through pairings, strains were chosen on the basis of their unique mitochondrial DNA haplotypes (Figure 6.16). Determination of the mitochondrial DNA haplotypes of 196 scorable hyphal-tip derived heterokaryons revealed several interesting features (Figure 6.18). The distribution of mitochondrial DNA haplotypes within the mycelia of the pairings examined is presented in Figure 6.19.

In compatible pairings between distantly related homokaryons, the mitochondrial genotype inherited belonged to that of the resident mycelium from which the sample was taken. However when the strains were closely related (F2-series and AF1.6), and the mitochondria only differed

Figure 6.14A Target, total genomic DNA samples (1) F2.9 and (2) F2.109, probed with variously treated F2.117 mitochondrial DNA or F2 fb nuclear DNA samples. (a) DNAseI treated F2.117 mtDNA, (b) DNAseI treated F2 fb nuclear DNA, (c) high salt, RNAse A treated F2.117 mtDNA, (d) high salt, RNAse A treated F2 fb nuclear DNA, (e) Low salt, RNAse A treated F2.117 mtDNA, and (f) Low salt, RNAse A treated F2 fb nuclear DNA.

(B) F2.103 target total genomic (tg) and nuclear (N) DNA samples treated with various agents prior to probing with F2fb mtDNA. (a) Uncut tg DNA, (b) EcoRV cut tg DNA, (c) Heat treated EcoRV cut N DNA treated with RNAse A under high salt conditions, (d) Unheated EcoRV cut N DNA treated with RNAse A under high salt conditions, (e) Heat treated EcoRV cut N DNA treated with DNAseI, (f) Unheated EcoRV cut N DNA treated with DNAseI, (g) Uncut N DNA. Long and short exposures are presented.

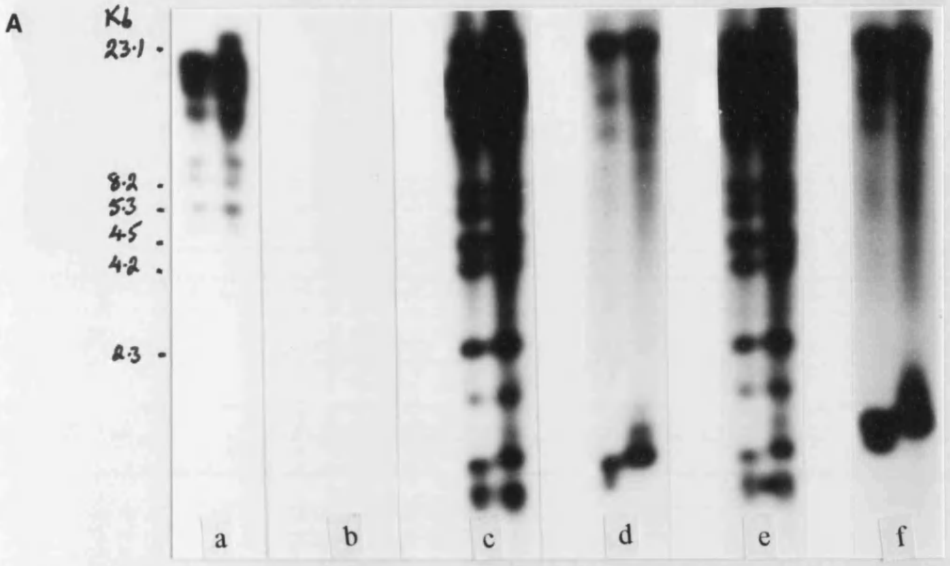


Figure 6.15. (a) Homokaryon F2.4, (b) homokaryon EP1.6, (c) homokaryon F2.4t, (d) fully compatible pairing between F2.4 and EP1.6, and (e) incompatible pairing between F2.4t and EP1.6. Treatment of homokaryons with rifampicin or tetracycline prior to pairing did not affect the outcome of the interactions.

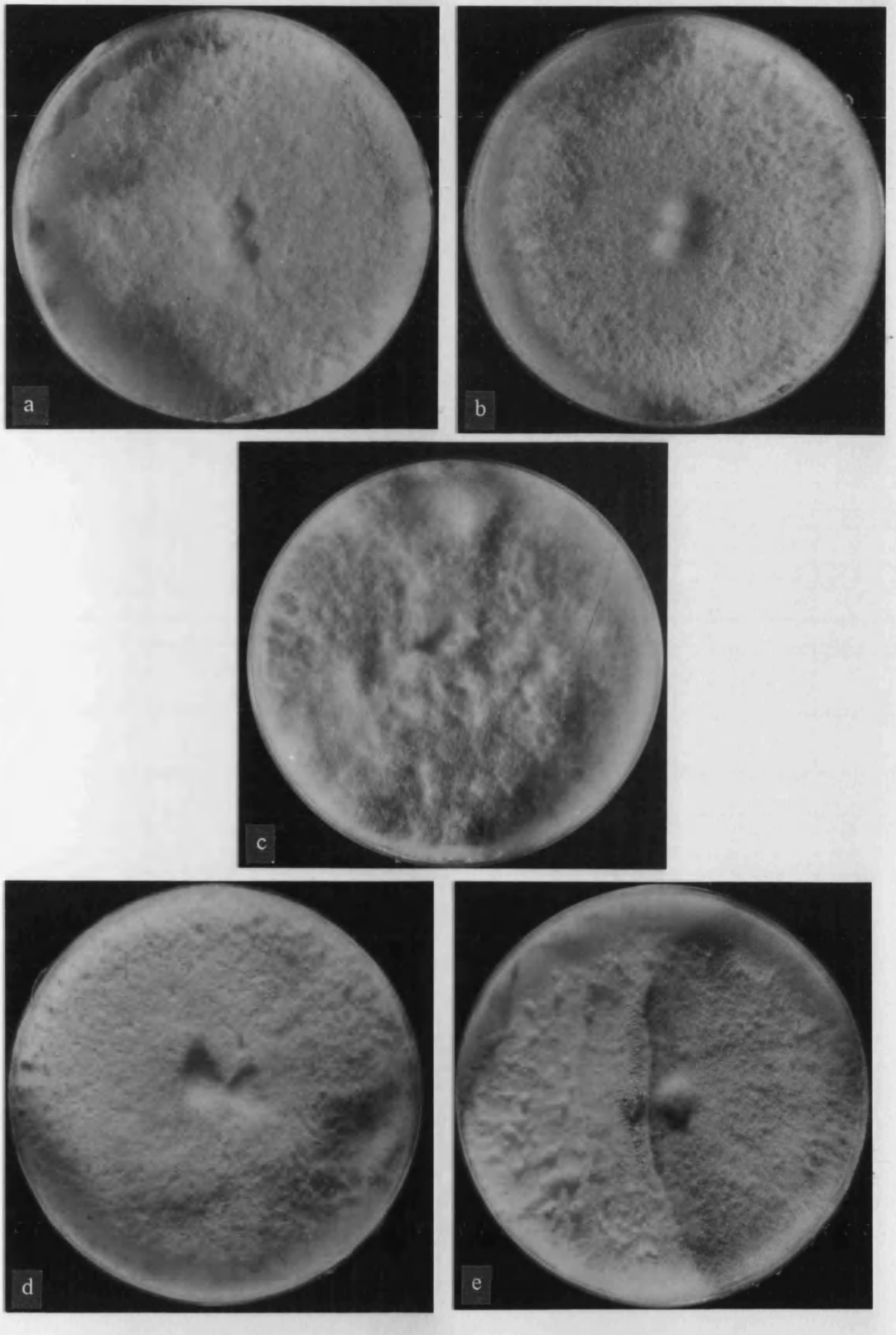


Figure 6.16. EcoRV generated mitochondrial DNA haplotypes of strains examined in this study; (a) profile of EP1 and EP1.7 strains, (b) profile of AF1.6, (c) profile of F2-series strains, and (d) profile of F2 homokaryons with 'altered' mating responses.

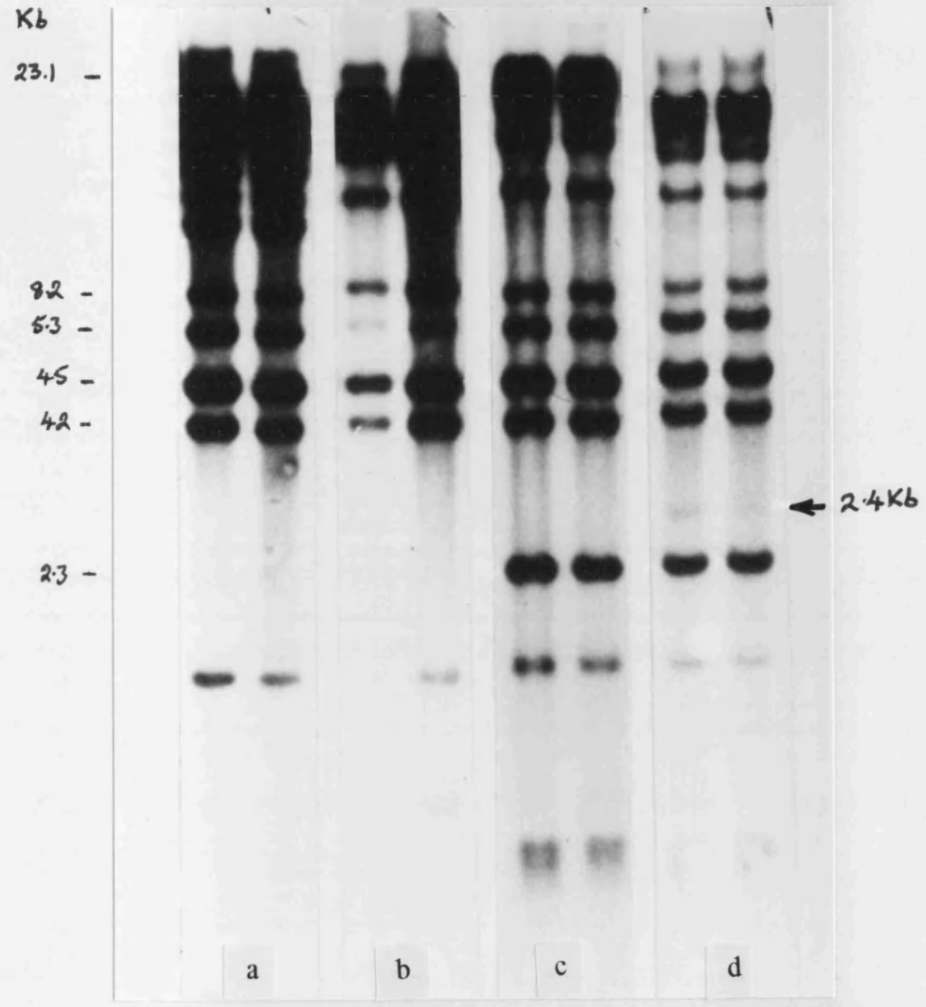


Figure 6.17. EcoRV digested (1) F2.9 and (2) F2.110 total genomic DNA probed with individual EcoRV fragments purified from low melting temperature agarose; (a) 8.1 kb fragment, (b) 6.7 kb fragment, (c) 5.7 kb fragment, (d) 4.5 kb fragment, (e) 4.2 kb fragment, and (f) 2.3 kb fragment, note the weak hybridization to the 2.4 kb additional band.

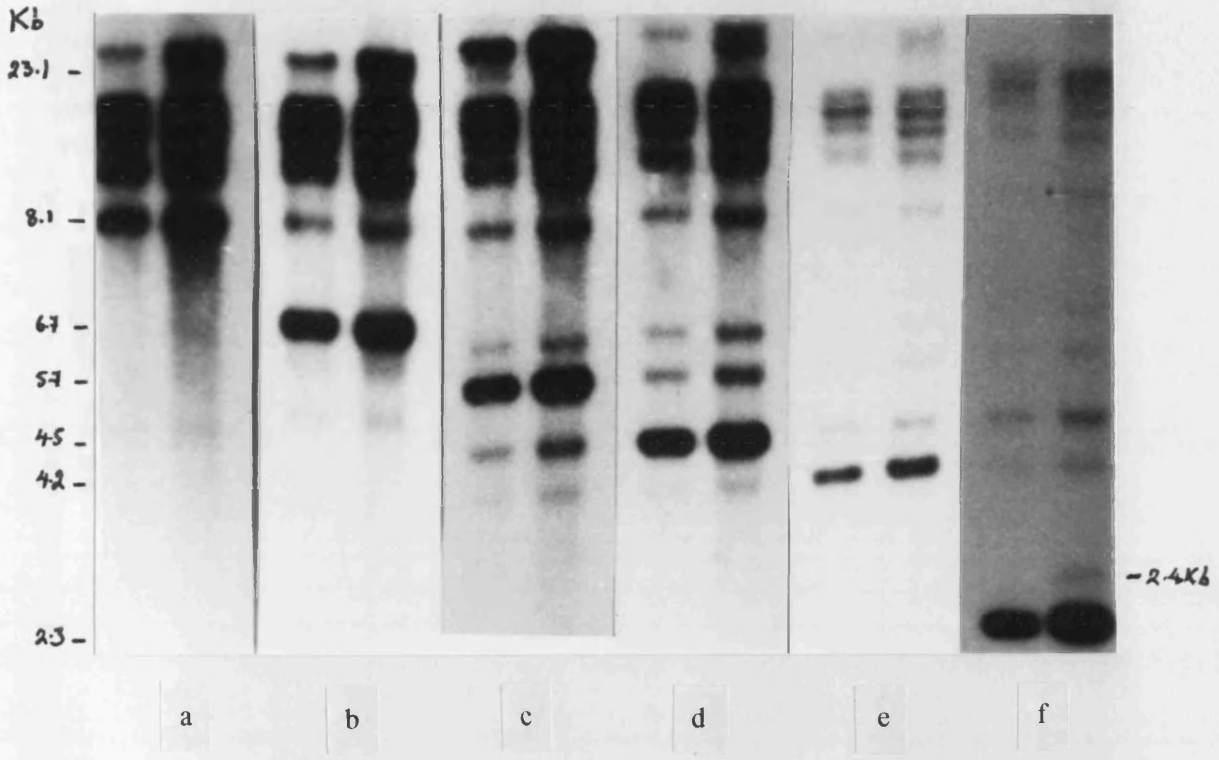


Figure 6.18. Mitochondrial DNA haplotypes of representative hyphal-tip progeny taken from various pairings between F2 and non F2-series strains highlighting (*) the transmission of mitochondrial DNA from one side of a pairing to another and the appearance of altered mitochondrial DNA haplotypes in some strains (arrowed). (a) AF1.6 x F2.10, lanes; (1) F2-side F2 mt, (2) F2-side F2 mt, (3) * F2-side F2 mt, (4) AF-side AF mt, (5) AF-side AF mt, (6) AF-side AF mt, (7) boundary F2 mt, (8) boundary zone F2 mt, (9) F2 side F2 mt, (10) * AF-side F2 mt, (11) F2-side F2 mt, (12) * F2-side AF mt, (13) F2-side F2 mt, (14) * AF-side F2 mt, (15) boundary zone F2 mt, (16) F2-side F2 mt, (17) AF-side AF mt, (18) * AF-side F2 mt, (19) boundary zone F2 mt, (20) F2-side F2 mt, (21) F2-side F2 mt, (22) AF-side AF mt, (23) boundary zone F2 mt (extra band) (24) boundary zone AF mt (25) F2-side F2 mt, and (26)* AF-side F2 mt. (b) EP fb x F2.4t, lanes; (1) boundary zone F2 mt, (2) F2-side F2 mt, (3) EP-side EP mt, (4) EP-side EP mt, (5) EP-side EP mt, (6) EP-side EP mt, (7) boundary zone EP mt, (8) boundary zone EP mt, and (9) boundary zone F2 mt.

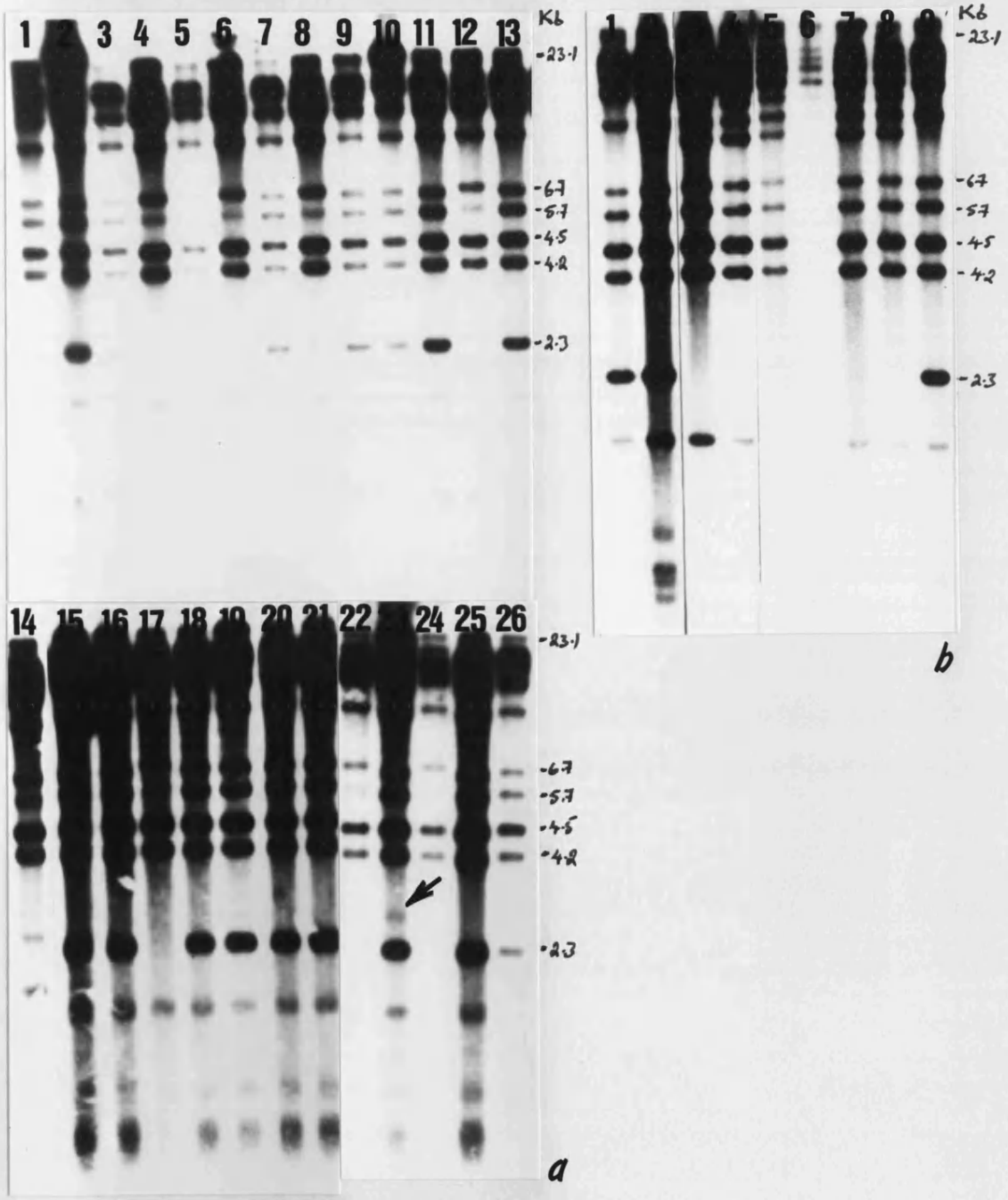
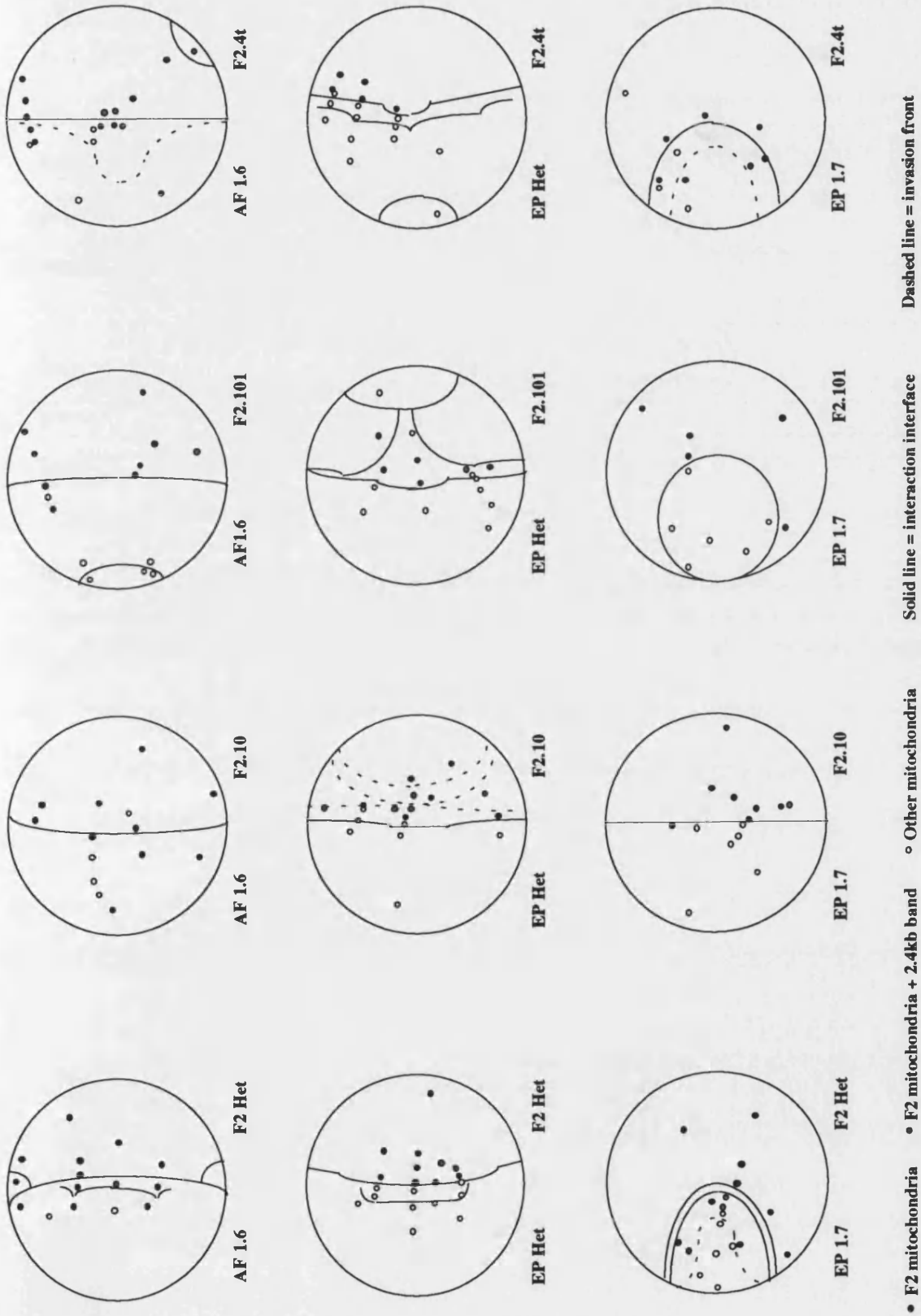


Figure 6.19. Summary diagram indicating the mitochondrial DNA haplotypes of hyphal-tip derived strains taken from pairings set up between F2 and non F2-series strains.



with respect to a single polymorphic EcoRV generated fragment, significant introgression of F2 mitochondrial haplotypes into non-self mycelia was detected.

In all of the pairings examined between homokaryons and heterokaryons, mitochondria from the heterokaryotic parent could be detected in mycelia derived from the homokaryotic side of the interactions. For example, in pairings between EP1.7 and F2 fb or F2.4t, mitochondrial introgression was restricted to the non-degenerate appression zone evident in the homokaryotic progenitor strain. No movement of mitochondria was detected in any of the pairings between heterokaryotic mycelia.

For those strains in which mitochondrial migration was observed, no mixed or heteroplasmic haplotypes were detected. In some cases degenerative hyphal-tip strains were obtained from pairings that upon subculturing, sectored to give different colony types with identical mtDNA haplotypes.

The novel 2.4 kb band found in F2 series progeny with altered somato-sexual recognition responses, was also detected in some of the hyphal-tip derived progeny generated in this experiment. In most cases, such haplotypes were detected in strains derived from locations adjacent to interfacial somatic incompatibility zones or pseudosclerotial plates.

DISCUSSION

The previously reported multi-allelic, unifactorial (bipolar) system of mating-type compatibility in *Stereum hirsutum* was upheld in this study. Several new observations however add to our understanding (or confusion) regarding nuclear exchange in this organism.

A reciprocal tendency to express the 'bow-tie' phenotype was noted between compatible and incompatible matings. In strains that had been stored for long periods, bow-tie formation was more prevalent than expected in incompatible matings, and less prevalent than expected in compatible pairings (cf Coates, 1984). Surprisingly, in strains that had been repeatedly sub-cultured, the reverse was observed; with more bow-ties expressed between incompatible partners than expected.

The control of bow-tie formation is thought to reside within a multi-allelic locus, the B-factor - which may be analogous to the B-mating-type factor of tetrapolar basidiomycetes (Coates & Rayner, 1985a). B-mating-type factor expression is constitutive in homokaryons (Raudaskoski, 1972; Richardson, 1993), and appears to be repressed in compatible matings during heterokaryon formation. A gradual loss of B-factor expressivity over time might therefore account for the patterns of bow-tie formation observed in the stored cultures. Furthermore, such a loss of B-factor expression may be correlated with a reduced rate of nuclear division (cf Raper, 1985).

According to a hypothesis outlined in a preceding section, B-mating-type factors promote mitosis during normal somatic development, but reduce it in compatible matings. Indeed a dual role of this nature has been recently demonstrated for a multi-allelic mating-type factor which promotes mitosis in haploids, and retards mitosis during mating, within the ciliate *Euplotes* (Vallesi *et al.*, 1995). Results described earlier would suggest that reduced rates of mitosis allow more extensive nuclear migration. A lower mitotic rate could therefore promote nuclear migration, even in incompatible matings; leading to more bow-ties than normal. Conversely, in fully compatible matings, heterokaryon formation might have been so rapid that no somatic incompatibility response was elicited whatsoever. This would then have prevented the expression of a bow-tie response.

Repeated sub-culturing may affect B-factor expression in a slightly different way, accounting for the reversal of the pattern observed in stored cultures. Repeated subculturing is associated with continuous growth, which must in turn be linked to sustained rates of nuclear division. Sustained nuclear division may be promoted further by the intermittent stresses imposed by mechanical injury associated with subculturing (cf final discussion on role of stress in stabilization). A high rate of mitosis may be associated with high levels of constitutive B-factor expression (which might be even higher than usual as a result of stress induced transposition events - see below. Earlier work on *Heterobasidion annosum* indicated that the lowest rates of migration were associated with highest rates of mitosis. Bow-tie formation in sub-cultured strains of *Stereum* could therefore be constrained to the immediate zone of contact in incompatible matings, which would then account for their low incidence.

Parag (1975) reported on the discovery of two cytoplasmic factors which affected mating responses in *Schizophyllum*. The F-factor was found in an attempt to isolate B-factor mutants, and produced similar morphological changes to those seen in the bow-tie responses of *Stereum*. Following two months in storage, segregants with the F-factor (flat) morphology returned to normal (including the expression of normal mating-type responses). The P-factor, which led to pseudoclamp formation in fully compatible matings, was epistatic to both the A and B-mating-type factors. Homokaryons with the P-factor were observed to produce clamps on their own - implying some similarity with regard to clamp-connection formation in *Stereum*. Following meiosis the role of the P-factor was observed to change, and was only operative in common-A matings.

The expression of the 'crescent' phenotype in some *Stereum* pairings may represent a restricted bow-tie response. Crescent formation was strongly associated with a specific C-mating-type factor (C1). Moreover two homokaryons, F2.4 and F2.101, both of which have shown altered mating responses, were the most likely to express this phenotype. The C-factor of *Stereum* controls mating-type compatibility *per se* and has both the A-factor and B-factor properties of tetrapolar basidiomycetes. Therefore, *Stereum* effectively has two unlinked loci with B-factor activity. Transpositional events between the two, perhaps associated with the changes described in mitochondrial DNA (associated with altered mating-type specificity)

might account for this transient phenomenon. Transposition could disrupt the stabilizing function of the A-factor component of the C-factor, leading to a loss of mitotic synchrony and to a loss of heterokaryon stability.

The altered mating-type specificities of some of the F2 strains examined were linked to an increase in size of a mitochondrial DNA fragment unique to the F2-series. It could not be ascertained whether the change involved a switch *per se* in the mating-types of the strains (each to a completely different specificity), or whether a switch occurred as a loss of all non-self recognition properties. The observed weak rejection of the parental heterokaryon and F2.4t lend some support to the second suggestion, though there are several precedents for mating-type interconversion in fungi.

Extensive studies of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, have revealed that mating-type switches are under the control of a complex genetic regulatory system (Klar, 1989,1992). In both cases, switching requires the transposition of a repressed, silent mating-type allele into an active mating-type cassette locus.

Reports of switching in filamentous fungi have been surprisingly limited - to two ascomycetes, *Chromocrea spinulosa* and *Sclerotinia trifoliorum* (Mathieson, 1952; Uhm & Fuji, 1983a,b) and one basidiomycete, *Agrocybe aegerita* (Labarére & Noël, 1992). Tentative claims for switching have also been reported in a *Glomerella sp.* (Perkins, 1987) and *Coprinus cinereus* (Puhalla personal communication to Brasier, 1985), but the situation in neither of these has been clarified.

In the tetrapolar agaric, *Agrocybe aegerita*, homokaryons are capable of switching mating-type specificities at both their A and B-factors during normal vegetative growth. Homokaryons obtained from a single dikaryotic isolate harboured a total of six different mating-types; two parental and four non-parental. Each homokaryon could switch to several different specificities, and in contrast to the situation in *Chromocrea* and *Sclerotinia*, switches were not unidirectional. Extensive genetic analyses indicated that the specificity to which a homokaryon could switch was fixed during meiosis in the parental dikaryon.

Labarére & Noël proposed a model that could account for the patterns of behaviour that they observed. The A and B-factors were said to reside in expression cassettes, with each haploid

genome containing two silent alleles for each factor. Wild-type homokaryons could therefore support two expressed, and four silent alleles. During meiosis in a dikaryon, each locus might be copied and transposed into one of the silent loci in its partners genome, simultaneously eliminating the silent copy. Switches in a homokaryon occurred when the expression cassette was reciprocally transposed within its own genome. Further experimental studies, implicated a chromosomal marking system, since in most cases the mobilized silent element was the one not involved in a transposition event during the preceding meiosis (cf single strand breakage model in *Schizosaccharomyces pombe* - Klar, 1987).

The unusual patterns of mitochondrial inheritance observed in this study as well as the changes in mitochondrial DNA associated with mating-type switches may be interlinked. The mechanisms responsible cannot be directly resolved from the available data, but the finding that mitochondrial DNAs from heterokaryons, and t-factor strains possess homology to repetitive elements in the nuclear genome; and that strains with additional mitochondrial DNA fragments had altered self-non-self recognition properties, may have particular significance.

One prediction of the genomic conflict paradigm is that mating-type genes may be responsible for the limitation of cytoplasmic and nucleo-cytoplasmic conflicts (Cosmides & Tooby, 1981; Hurst, 1992). Following the establishment of a stable heterokaryon, cytoplasmic elements may be altered by the mating-type loci so that they no longer exhibit antagonistic properties. Consequently when the cytoplasms of a homokaryon and a heterokaryon are brought into contact; the mitochondria of the heterokaryon might be expected to invade the homokaryon, - passing unnoticed.

Mitochondrial DNA of *Stereum* is highly variable both within the wider context of a population, and in terms of variation within individuals. This variation does not appear to affect normal cellular functions since all strains are competent in their own right. Much of the variation would therefore appear to be redundant. The polymorphisms involved may however play a role in recognition phenomena at the population level. Geographical differentiation of the loci responsible for attenuating conflicts is apparent in this study, since in a sympatric context, where the mitochondria were largely homologous, mitochondrial migration was observed,

whereas in an allopatric context, no mitochondrial exchange was seen. The significance of the patterns of mitochondrial inheritance observed in *Stereum* will now be placed in perspective.

Following the conjugation of two mating-type compatible fungi, mitochondria are generally inherited in a bilateral, uniparental fashion (the different mycelia that come together retain their resident mitochondrial populations). This pattern has been found in most ascomycetes examined, including *Aspergillus nidulans* (Rowlands & Turner, 1976) and *Neurospora crassa* (Mitchell & Mitchell, 1952; Mannella, Pittenger & Lambowitz, 1979); as well as basidiomycetes such as *Agaricus bitorquis* (Hintz, Anderson & Horgen, 1988), *Armillaria bulbosa* (Smith *et al.*, 1990), *Coprinus cinereus* (Casselton & Condit, 1972; Baptista-Ferreira, Economou & Casselton, 1983; May & Taylor, 1988), *Schizophyllum commune* (Specht, Novotny & Ullrich, 1992), *Stereum hirsutum* and *S.complicatum* (Ainsworth *et al.*, 1992).

In addition to the results presented here however, a considerable body of work questions the widespread belief that bilateral uniparental inheritance is the only pattern of mitochondrial transmission in fungi. For example some early work on cytoplasmic inheritance demonstrated the occurrence of heteroplasmons, or mixed cytoplasms when extranuclear mutants were observed to complement one another in *Neurospora crassa* (Pittenger, 1956; Bertrand & Pittenger, 1972). The general validity of their observations might be questioned since the strains examined were nearly isogenic with respect to their cytoplasms. However, other reports are more conclusive.

Uniparental replacement of mitochondria has been observed to occur over short distances in *Schizophyllum commune* (Watrud & Ellingboe, 1973a,b), and in *Neurospora crassa / N.sitophila* hybrids (Reich & Luck, 1966). Ultrastructural investigations of hyphal anastomoses between vegetatively compatible and incompatible strains of *Cryphonectria parasitica* also revealed mitochondrial migration, though this was limited to degenerate regions (Newhouse & MacDonald, 1991). Sadly, molecular RFLP studies failed to confirm their observations (Gobbi *et al.*, 1990). Tentative claims for mitochondrial transfer have also been made for *Ustilago violacea* (Wilch, Ward & Castle, 1992) and with more certainty during the formation of unstable heterokaryons in *Neurospora crassa* (Collins & Saville, 1990). Gordon & Okamoto (1992) also inferred that mitochondrial transfer must have occurred between

weakly incompatible strains of *Fusarium oxysporum* in order to account for the distribution of mtDNA haplotypes in natural populations. Studies on *Neurospora tetrasperma* have perhaps provided the strongest evidence to date for mitochondrial migration following mating in a filamentous fungus (Lee & Taylor, 1993).

Lee & Taylor (1993), observed four different patterns of nuclear migration when they paired together homokaryons derived from the pseudohomothallic ascomycete *Neurospora tetrasperma*. These were; (1) no migration, (2) unilateral migration, (3) bilateral migration, and (4) mutually exclusive unilateral nuclear migration. In replicates of pairings exhibiting mutually exclusive nuclear migration a strain might act as a donor (male) in one replicate, and as an acceptor (female) in another. Mitochondrial transmission in the first three categories conformed to the normal bilateral, uniparental inheritance pattern as expected, however, in the mutually exclusive pairings, mtDNA from the acceptor (female) mycelium replaced mtDNA of the donor (male) strain, regardless of which strain was acting as the acceptor or donor. Additional studies also indicated that the acceptor mycelia were heterokaryotic for mating-type and that the donor mycelia remained homokaryotic.

Patterns of mitochondrial inheritance in the majority of isogamous eukaryotes including *Didymium iridis* (Silliker & Collins, 1988), *Physarum polycephalum* (Kawano *et al.*, 1987; Kawano & Kuroiwa, 1989; Meland *et al.*, 1991), *Polysphondylium pallidum* (Mirfakhrai, Tanaka & Yanagisawa, 1990), and *Chlamydomonas reinhardtii* (Boynton *et al.*, 1987) are uniparental. Uniparental maternal inheritance is also the normal state of affairs in organisms that produce anisogamous gametes. Of course, exceptions to the rule can be found, which include the complete replacement of one mtDNA population by another in heteroplasmic flies of *Drosophila melanogaster* (Niki, Chigusa & Matsuura, 1990) and the frequent replacement of mitochondrial populations in maternal lineages of *Drosophila simulans* (Satta *et al.*, 1988) and cattle (Koehler *et al.*, 1991). The relatively high frequency with which heteroplasmy occurs in the *Drosophilids* may be attributed to the immense size of their sperm. For example in *Drosophila bifurca*, sperm cells may be up to 20 times longer than the male flies that manufacture them, reversing the traditional pattern of anisogamy (Pitnick, Spicer & Markow, 1995).

Numerous hypotheses have been proposed to account for uniparental mitochondrial inheritance. The simplest of these involves monogametic organelle transmission in which organelles from one parent are excluded from the zygote. This mechanism is limited to a relatively small number of taxa (Mogensen, 1988). A related hypothesis, proposed by Van Winkle-Swift (in Silliker & Collins, 1988) suggests that uniparental inheritance could arise from the clustering of mitochondria around nuclei. Very little work has been performed to investigate this possibility further. However, evidence in its favour is provided by the observation that some cytoplasmic characters in yeast, *Neurospora* and *Aspergillus* segregate with specific nuclei in mycelia, conidia or ascospores (Brewer & Fangman, 1980; Srb, 1958,1963; Roper, 1958).

Random drift was proposed by Birky (1983) as the dominant factor influencing transmission in those organisms that generate zygotes from the fusion of anisogamous gametes. Maternal cells often contain thousands of copies of mitochondrial DNA and paternal cells relatively few. Since the rate of random drift is expected to be linked to the number of organelle copies per cell, drift can only account for uniparental inheritance in cells that contain fewer than 100 copies of the rare organelle (Birky, 1983). Silliker & Collins (1988) calculated that random drift would require at least 120 cell division generations to achieve 90% segregation of mitochondrial genotypes for a cell starting with only 60 mitochondrial DNA molecules. Their calculations demonstrate the inadequacy of such a model operating alone, to account for patterns of inheritance typically observed. Random drift cannot adequately account for patterns of uniparental inheritance in most organisms that produce isogamous gametes or somatic heterokaryons.

Mitochondrial input bias, can however, account for uniparental mitochondrial transmission in yeast (Birky *et al.*, 1978). Dujon (1981), found that only 20 cell division generations were required for an initially heteroplasmic diploid yeast cell to segregate into homoplasmic lines. In temporarily heteroplasmic cells, differential replication might therefore affect the final proportions of the mitochondrial genotypes in subsequent cell lines or ultimately lead to the fixation of one type. The success of a genome may be intrinsic; for example smaller mitochondrial genomes replicate more rapidly and consequently attain dominance in crickets

(Rand & Harrison, 1986). A replicative advantage for a mitochondrial genotype contributing towards its biased inheritance has also been described for *abn-1* mutants in heteroplasmons of *Neurospora crassa* (Diacumakos, Garnjobst & Tatum, 1965)

Selective advantages for specific cytoplasmic genotypes might also lead to their predominance under the appropriate conditions. For example, antibiotics have been used in many studies to select for increased numbers of mitochondria containing resistance markers (eg Thomas & Wilkie, 1968; Linnane *et al.*, 1968; Adouette & Beisson, 1972). However, apparently non-advantageous mitochondrial genomes lacking oligomycin resistance markers were preferentially inherited at the expense of mitochondria known to be resistant to oligomycin in *Aspergillus nidulans* (Mason & Turner, 1975), which would suggest that selection is not always relevant.

A popular, though largely unsubstantiated alternative explanation to these ideas, is the genomic restriction hypothesis. Here, one population of mitochondria is thought to be marked for destruction prior to cell fusion (Sager & Lane, 1972; Meland *et al.*, 1991). Some support for this theory was provided by Birky (1978), who directly observed the destructive elimination of paternal mitochondria at an early stage of zygote formation in some mammals and algae. Observations on the inheritance of chloroplast DNA (ctDNA) in *Chlamydomonas reinhardtii* (Sager & Ramanis, 1973; Sager, Sano & Grabowy, 1984; Kuroiwa, 1985), led to the proposal that nucleases might selectively destroy the chloroplast genomes of one parent and not the other producing uniparental inheritance. Charlesworth (1983), and later Boynton *et al* (1987), extended the active destruction hypothesis by suggesting that the ctDNA of one parent might be methylated, so allowing the ctDNA to avoid degradation by methylation sensitive nucleases. Some evidence for ctDNA methylation in *Chlamydomonas* has been obtained (Sager & Ramanis, 1973), but the methylation status of specific ctDNA populations did not appear to affect the patterns of chloroplast inheritance (Bolen *et al.*, 1982).

The methylation status of mitochondrial DNAs of the *Stereum* isolates used in this study were ascertained using the restriction enzymes *HpaII* and *MspI*. No methylation, either before or after heterokaryon, formation was detected. Indeed, no evidence for mtDNA methylation has been found in any species examined (Beckers *et al.*, 1991). However, both the low levels of

methylation thought to be necessary to mark a population, and the narrow time scale over which organelles need to be marked, may preclude detection using such an approach.

Both classical and molecular genetic studies have provided strong support in favour of the widespread occurrence of recombination between mitochondrial DNA molecules, particularly at the interface between fungal mycelia where cell fusions are repeated many times (Earl *et al.*, 1981; Croft & Dales, 1984; Perkins & Turner, 1988). Transient mitochondrial fusion might therefore allow the transmission of mobile genetic elements between mitochondrial populations, opening up the possibility for conversion of one mitochondrial population into another. Such behaviour has been observed for the *omega* and *var1* elements of yeast (Dujon, Slonimski & Weill, 1974; Butow, Perlman & Grossman, 1985; Zin & Butow, 1985) and for a 1 kb insertional element in *Chlamydomonas* (Boynton *et al.*, 1987). Rapid isogenization of mitochondrial populations as a result of gene conversion has also been described in *Saccharomyces cerevisiae* (Birky, 1976) and in *Aspergillus* (Rowlands & Turner, 1974).

In these reports there are many parallels with the situation that has been described in *Stereum*, but none can entirely account for all of the patterns observed. Whatever the nature of the mechanism responsible for mitochondrial transmission in *Stereum*, it is important to bear in mind the role played by nucleo-cytoplasmic interactions, particularly with regard to the differences that were observed between mated and unmated strains.

A strong link between mating-type and the inheritance of organelles has been observed in many species. Surprisingly different rules appear to operate for mitochondria and chloroplasts as the following examples illustrate.

Pairing experiments in *Physarum polycephalum* have revealed a strong correlation between the mating-type (*matA*) alleles possessed by interacting haploid amoebae, and the final genotype of the mitochondria inherited by the plasmodium. Mitochondria were transmitted in accordance with a pattern dictated by the mating-type with which they had been associated, prior to mating. Moreover, the mating-types (of which 13 are known), could be ranked into a linear dominance hierarchy, such that mitochondria associated with mating-types with a high ranking, displaced mitochondria associated with mating-types with a lower ranking (Kawano *et al.*, 1987; Kawano & Kuroiwa, 1989). The mitochondrial genotype itself was completely

irrelevant to the patterns of transmission observed, as was the identity of the two other loci responsible for mating in *Physarum*, *matB* and *matC*.

When closely related strains of the cellular slime mould *Polysphodylium pallidum* were paired together, mitochondria were inherited preferentially from the *mat2* parent rather than from the *mat1* parent (Mirfakhrai, Tanaka & Yanagisawa, 1990). In a small proportion of pairings between distantly related strains mitochondria transmitted from the *mat1* parent. This again indicates the importance of geographical differentiation in the loci responsible for regulating genomic interactions and that these loci may be dysfunctional in an allopatric context.

In *Chlamydomonas,* meiotic progeny from a cross normally receive their mitochondria from the mt^- parent (Boynton *et al.*, 1987), and their chloroplasts from the mt^+ parent (Sager & Lane, 1972; Grant, Gillham & Boynton, 1980; Boynton *et al.*, 1987). Gamete fusion in *Chlamydomonas* is often followed by chloroplast fusion, which is then followed by the destructive elimination of both sets of ctDNA (in some cases as much as 95% of the ctDNA can be lost). Typically the ctDNA inherited from the mat^+ parent is degraded more slowly than that from the mat^- parent, resulting in its final dominance (Chiang, 1976).

Fusion studies of vegetative diploids of *Chlamydomonas* have indicated that organellar inheritance can, at least initially, be biparental (Matagne & Hermesse, 1980). In subsequent mitotic divisions, organelles then segregate at random to restore a homoplasmic condition. The exchange of organelles between previously mated strains of *Chlamydomonas* may be analogous to the introgression of mitochondria from heterokaryons into homokaryons in *Stereum.*

Several other studies on fungi have established a relationship between mating-type and mitochondrial inheritance. In the heterobasidiomycete *Ustilago violacea*, meiotic progeny sets expressing the a_1 mating-type contain mitochondria from either of their parental strains, whilst those with the a_2 mating-type almost exclusively contain mitochondria from the a_2 parent (Wilch, Ward & Castle, 1992). This might reflect the differences in activity of the *a1* and *a2* mating-type genes at different stages of the cell cycle that were highlighted by Day & Cummings (1975). Studies of the bifactorial (tetrapolar) basidiomycete *Schizophyllum*

commune also indicated that mitochondrial inheritance can be directly influenced by the activity of the mating-type alleles (Watrud & Ellingboe, 1973a,b). In common-A, common-AB and fully compatible matings, mitochondrial transfer was observed over a distance of at least 4 cells, or 1.2 cm beyond the interaction zone, whereas no migration was evident in the localized heterokaryons generated in common-B matings. Transmission was also apparent in di-mon pairings. The occurrence of mitochondrial migration in common-AB pairings in the absence of nuclear migration strongly suggests that the two events may be independent.

The B-factor locus not only affects nuclear division rates, but also mitochondrial division rates. Casselton & Economou (1984), showed that B-factor mutants of *Coprinus cinereus* often contained smaller mitochondria than their normal monokaryotic counterparts. In prokaryotes, cell size is generally linked to division rate; such that slow division rates are associated with small size (Schaechter, Maaløe & Kjeldgaard, 1958; Helmstetter & Cooper, 1968). Slow replication rates amongst mitochondria might permit introgression, since the conflicts associated with replication (see previous section on nuclear migration) would be avoided. Changes in B-factor expression during heterokaryosis might therefore account for the transfer of mitochondria from heterokaryons to homokaryons in *Stereum.*

Taken together the observations presented here, and elsewhere in the literature, suggest that organelle transmission is controlled by genes at, or near, the mating-type locus. The genes responsible may be silenced through mutation, and can switched off or modified in vegetative diploids or heterokaryons. Furthermore genetic disparity, at loci not related to mating-type can allow other patterns of mitochondrial inheritance to emerge. To date, it has not been clearly established whether the mating-type loci themselves are responsible for the observed behaviour of organelles, or whether other loci tightly linked to them are involved. The complete map of yeast chromosome III may reveal some candidates around the mating-type locus, including the centrally important cell cycle control gene, *cdc25*, which is adjacent to the expressed mating-type locus (Oliver *et al.*, 1992).

Pertinent information regarding the nature of the unique 2.3kb fragment found in the F2-series strains has come to light since the submission of this thesis (Zac Watkins, John Beeching; Jackie Constable; Alan Rayner - personal communication). The full DNA sequence of this fragment has been deduced. Comparisons with sequence databases have indicated that the fragment contains a terminal region with significant homology to a class of DNA polymerases that are found in some mitochondrial genomes and linear mitochondrial plasmids. The existence of a polymerase in this apparently 'non-essential' fragment, and its hybridization to a 2.4kb fragment in strains with altered somato-sexual recognition properties may be highly significant. The new findings lend additional credence to the proposition that mating-type responses may involve some manipulation / regulation of mitochondrial replication as a means of overriding cytoplasmic conflict.

SECTION VII

FINAL DISCUSSION: GENOMIC CONFLICT AND DISPARITY WITHIN FUNGAL MYCELIA

Final discussion

The data outlined in this thesis are consistent with the existence of genomic conflicts within laboratory-synthesized heterokaryons of *Heterobasidion annosum* and *Stereum hirsutum*, and a loss of conflicting partnerships in natural populations. The corollary of such a finding is that interactions between genomes in the sympatric context are subject to unremitting selection pressures which directly remove conflicting associations from the population and also that co-evolutionary feedbacks operate within a population to modify genetic exchange systems. The existence of regulatory loci that moderate patterns of genetic exchange , and perhaps counteract conflicts is supported by the finding that allopatric matings result in completely different modes of heterokaryon emergence and stabilization when compared with sympatric matings.

In contrast to competition, conflict has largely been neglected as a major force in evolutionary biology. This is surprising, since to appreciate the creative might of conflict in an evolutionary arena, we have only to take a look at our own recent social and political history. Conflict within and between nations, peoples and individuals, has perhaps been the most potent element changing and shaping the society in which we live.

An extreme manifestation of such conflict is a war of attrition, in which neither combatant achieves dominance. Time, materials and lives, may be invested in an attempt to attain goals that dwindle in significance with the passage of time (a consequence of the law of diminishing returns). A major source of innovation arising from such a conflict is the inevitable arms race that occurs in the search for new technologies with which to disable, disarm, or destroy opposing forces. Conflict of this nature is extremely damaging to the participants involved, since it inexorably leads to mutual degeneration.

Incompatibility, in the biological sense, is analogous to a war of attrition, in which the only stable outcomes are degeneration / death, or perhaps divorce. Neither of these outcomes permit significant evolutionary change since the *status quo* is maintained. Incompatibility is therefore largely an all or nothing affair, its consequences are immediate, with little scope for resolution.

In many ways, wars of attrition are less important as mediators of change than conflicts that can be resolved. Conflict resolution may either occur through a rise in dominance of one of the warring factions, or alternatively, through compromise - perhaps as a result of the intervention of a moderating third party. In such a scenario, the repercussions may be global, as well as local, leading to new policies, new laws, the coalescence or separation of peoples, and the definition of new geographical boundaries. Conflict resolution can therefore provide a window of opportunity that leads to the emergence of a variety of stable and metastable states through a combination of suppression, subjugation, coercion, and dominance. Such an array of possibilities clearly separates conflict from the narrower issue of incompatibility, and establishes conflict as the more productive source of evolutionary innovation.

Incompatibility *per se* is of clear relevance to organisms that sequester their sexual functions within specialized cells, or which limit their genetic contribution to a small number of nuclei. It is therefore pertinent to consider 'incompatibility' when studying fungi such as the ascomycetes. Incompatibility may not however be an accurate descriptor for the range of interactions possible when populations of nuclei of disparate origin are associated within a common cytoplasmic domain. Since 'incompatibility' may not be expressed in such a situation (according to the ability of the associated genomes to resolve their differences) the term conflict may be more appropriate. In contrast to many of the instances of 'incompatibility' highlighted in the literature, the conflicts described in this study are not necessarily, all or nothing events, rather they encompass the wider range of context dependent alternatives just described.

A consideration of the options available to fungi to attenuate conflict may provide a strong foundation upon which to base future studies in the new discipline of genomic conflict. Conflict resolution in a biological sense requires the co-operation of a range of processes functioning at many levels of organization. Such processes may; (i) minimize the disruption of nucleo-cytoplasmic partnerships (perhaps accounting for the predominance of uniparental, maternal inheritance); (ii) promote the onset of diploidy (constraining patterns of gene

expression and inducing parasexuality); (iii) compartmentalize mating (spatially separating the genomes); (iv) promote interference (destroying invaders leading to outright supremacy) or over-replication (out-numbering partners leading to dominance); (v) or silence partners (coercion through epigenetic modifications). Finally, (vi) frequency dependant selection operating at the population level, may dictate an evolutionarily stable strategy - perhaps sub-dividing a population and ultimately leading to speciation events.

Some of these will now be considered in more detail, taking examples from a variety of mycological and non-mycological sources, to back up the propositions.

Cellular stress and stabilization

Stabilization of genomic associations requires the attenuation of developmental incompatibilities. Such attenuation may be genetically programmed, or may result from internally driven, context-dependant and therefore to a large extent (serendipitous) feedback processes leading to developmental integration.

Heterokaryosis necessitates the juxtaposition of two disparate genomes or different sets of molecular machinery in the same cell. These machineries regulate fundamental cellular processes, including those responsible for transcription, translation and replication. Associations may therefore generate a state of tension between the partners if the complex conditions required to interface these machineries are not fully resolved. As a consequence, organisms open to non-self access, must utilize their relatively static genetic information, (genes), in a dynamic fashion implementing both epigenetic and hyperepigenetic shifts.

The cellular stress response in mammalian cells provides a good example of genomes, evolutionarily adjusted to 'normal' cellular functions, responding in a dynamic fashion when their molecular machineries are disrupted (Herrlich, Ponta & Ramsdorf, 1992). When mammalian cells are perturbed by a variety of agents, such as oxygen free radicals (Cerutti, 1985; Storz *et al.*, 1990), heavy metals (Caltabiano *et al.*, 1986; Shelton, Todd & Egle, 1986),

or heat (Lindquist, 1986) a cascade of signal transduction pathways are elicited which lead to changes in the behaviour of pre-formed replication factors, transcription factors and RNA processing enzymes. The changes that occur include increased rates of DNA replication, prolonged mRNA half-lives, increased translation rates and altered protein stabilities. Such gross effects are similar to changes that have been described during and after mating in slime moulds (Schrauwen, 1985), and some true fungi (Wang & Raper, 1970; Ross, Martini & Thoman, 1973; Moore & Jinjis, 1981) so the following discussion of the cellular stress response in mammals may have some significance to our understanding of fungal biology, and in particular the override mechanism hypothesized by Rayner *et al* (1984).

Many of the transcription factors induced by cellular stress in mammalian cells are transforming proteins, encoded for by nuclear oncogenes. The synthesis and activity of one of these transforming proteins, AP1 (*c-fos, c-jun*), indicates that once a cellular stress response has been initiated, and in the absence of any further assault, its activity may be down-modulated. In part, this can be attributed to the progressive phosphorylation of the components of AP1 (*c-fos, c-jun*), leading to a reduction in its activity (Curran *et al.*, 1984; Kovary & Bravo, 1991). Competition between AP1 and other transcription factors affects the overall patterns of cellular gene expression (Kerr, Miller & Matrisian, 1990) with the final outcome, being determined by the relative abundance of opposing factors (Herrlich *et al.*, 1992).

The signalling pathways elicited by DNA damage were observed to become refractory to repeated induction in mammalian cells when they were compromised further by the same agents (Buscher *et al.*, 1988). However other agents could stimulate additional rounds of activity in the same metabolic and/or genetic pathways given the opportunity.

These observations may provide some insight into the events that occur during self / non-self interactions in higher fungi. Genomes within homokaryons might be co-adapted to their own sets of transcription factors and regulatory proteins; the mixing of different populations of nuclei could disrupt the balance, initiating pathways analogous to those described in the

cellular stress response. This contention is supported by the finding that following the fusion of irradiated and non-irradiated mammalian cells, the heterokaryotic cells that arise exhibit patterns of transcription and translation indicative of a damage response (Nomura & Oishi, 1984; Lücke-Huhle & Herrlich, 1987). These pathways could be blocked by protein kinase inhibitors, suggesting the involvement of cellular signalling molecules (Buscher *et al.*, 1988; Kramer *et al.*, 1990).

Since the key elements responsible for transcription, translation and replication need to be synchronized in a stable heterokaryon it might be expected that pathways devoted to intracellular / intergenomic signalling and cell cycle regulation will be initiated early on in an association. Successful conjugation and diploid formation in two yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* requires the arrest of nuclei in the G1-phase of the cell cycle, which is mediated by the mating-type specific pheromones (Staben, 1995). The pheromonal components of the mating-type factors of ascomycetes and basidiomycetes may also be acting in such a manner, co-ordinating the synchronization of cell cycle activities.

Delays in the attenuation of association induced stress, may engender additional conflicts. A continued input of stress inducing factors (non-self genetic information), may lead to unresolvable cellular instabilities and ultimately degeneration. Patterns of intracellular degeneration within interaction zones of various fungi have been described that are clearly reminiscent of programmed cell death or apoptosis (Ainsworth *et al.*, 1990b), which is the ultimate consequence of prolonged cellular stress in mammalian cells.

Genomic silencing

Epigenetic silencing of one genome in an association, possibly through mechanisms similar to those described for imprinting or chromosome inactivation may be another solution to the problem of conflict.

Any element, functional group, nucleic acid or protein that can interact with a gene and influence its pattern of transcription, provides the basis for the generation and maintenance of

epigenetic variation (Jablonka & Lamb, 1995). Furthermore, mechanisms promoting such variation have the potential to silence genomes.

Differences between active and inactive chromatin have long been recognised. During interphase, chromatin is typically subdivided by cytogeneticists into dense heterochromatin and dispersed euchromatin. Most (constitutive) heterochromatin contains little genetic information and is largely structural rather than informational. A small proportion of (facultative) heterochromatin is only found in cells at specific stages of their development. A classical example of the latter is the inactive X-chromosome in females of mammals which is condensed at an early stage of development to form a Barr body. This inactive state is maintained through subsequent clonal generations, possibly to maintain the same dose of X-linked genes in males (XY) and females (XX). Silent X-chromosomes are then reactivated in the germ line.

Brown *et al* (1991) described a centre of transcription on inactivated X-chromosomes, *Xist*, which acted as a 'global' repressor. Although this element acted in a *cis*-fashion, similar elements might operate in a *trans*-fashion, affecting the state of expression of entire chromosomes; and maybe whole genomes.

There is restricted evidence to support the conjecture that genomic silencing occurs within fungal mycelia. However, genomic imprinting has been implicated as a determinant of mating-type stability and inheritance in *Schizosaccharomyces pombe* (Klar, 1987), and differential patterns of centromeric methylation have been detected in monokaryons and dikaryons of *Coprinus cinereus* (Zolan & Pukkila,1986). Other workers have detected differences in the methylation pattern of rDNA repeats in *Neurospora* (Russell *et al.*, 1987a,b) and *Schizophyllum commune* (Buckner, Novotny & Ullrich, 1988).

Modifier genes are thought to be able to account for variations in the penetrance of dominant alleles in mice, eg *fused* is more penetrant when maternally derived than when paternally derived as a result of interactions between modifiers of heterochromatization (Azulnik & Ruvinsky, 1988). Moreover, Allen *et al* (1990) reported that transgenes could become locked

into an unexpressed state following repeated passage through the maternal line of a specific mouse strain (BALB/C). This was accompanied by a stepwise increase in the methylation state of the gene. Similar phenomena operating within fungal heterokaryons, might not only reduce conflict, but could also form the basis of an epigenetic cellular memory system that could account for many of the other patterns of behaviour highlighted in this thesis.

Such an inheritance system might partially explain why the homokaryons recovered from heterokaryons often retained some of their heterokaryotic properties. Metabolic or physiological disruptions of the type mentioned earlier with respect to cellular stress, are another aspect of circumstance that promotes diversification, through hyperepigenetic, rather than epigenetic means. Hyperepigenetic inheritance systems ensure that 'organizational' rather than 'developmental' drives predominate, resulting in a strong tendency for fungi to exhibit 'alternative phenotypes' (Rayner, Ramdsale & Watkins, 1995). In indeterminate systems, innovation and speciation, in contrast to determinate systems, proceeds via 'the bifurcation of developmental programs, not necessarily with the branching of phylogenetic trees' (West-Eberhard, 1986).

Promotion of diploidy

During the establishment of a new association, diploidy might arise as a means of limiting conflict and developmental incompatibility since the control of cellular functioning is placed under a single regulatory umbrella. Furthermore, diploidy in conjunction with a parasexual cycle might bring about the elimination of conflicting genetic information.

Observations on nuclei of wheat-barley hybrids (Heslop-Harrison *et al.*, 1990; Leitch *et al.*, 1990) indicated that genomic partitioning could occur within a diploid. The chromosomes of one parent resided at the periphery of the nucleus where there was less transcriptional activity. As a result, the phenotype of the plants was largely determined by one parent, rather than both.

This is clearly a simple mechanism that would avoid the expression of developmental incompatibilities.

Genomic disparities may engender structural as well as informational conflicts which might provide a strong selection pressure for mechanisms that favoured the elimination of conflicting material. The parasexual cycle may be just such a defence mechanism. It would therefore be expected to operate at a higher frequency in genomic associations that were most likely to be in conflict, eg allopatric combinations or in incompatible matings. Various studies on interspecific hybrids of *Aspergillus* (Anné, Eyssen & De Somer, 1976; Anné, 1982; Ferenczy, 1976,1981) and *Stereum* (Ainsworth *et al.*, 1992) would suggest that this is indeed the case. Furthermore unstable diploids are produced at higher frequencies during illegitimate matings than in fully compatible matings eg *Coprinus lagopus* (Casselton, 1965; Day & Roberts, 1969; Swiezynski, 1962).

Some of the most remarkable examples of genomic re-assortment, linked to parasexual processes have been described in haploid-diploid matings of various *Armillaria* species. The most frequent outcome is the simple replacement of the haploid by the diploid (Carvalho *et al.*, 1995), however, in some pairings a mosaic of dikaryons forms. This might arise through the haploidization and re-assortment of the diploid nuclei, followed by the segregation of recombinants with haploid nuclei from the monokaryon. Moreover, in such pairings, the haploid monokaryon also appeared to undergo a variety of genetic alterations. Another outcome that was observed, albeit rarely, resulted in the formation of monokaryotic cells in which all of the molecular markers from the diploid and haploid parents were present - a condition consistent with triploidy (Carvalho *et al.*, 1995). This outcome was not however observed in *A.ostoyae* (Rizzo & May, 1994) and therefore awaits confirmation.

Parasexuality has been demonstrated within a wide range of ascomycetes and also in some imperfect fungi (Fincham, Day & Radford, 1979). The operation of a parasexual cycle in the basidiomycetous fungi has, in comparison, been a poorly documented phenomenon.

Nonetheless, several convincing reports have been made for a variety of species including *Agrocybe aegerita* (Marmeisse, 1991), *Coprinus radiatus* (Prud'homme, 1970; Swiezynski, 1962,1963), *Schizophyllum commune* (Crowe, 1960; Ellingboe, 1963; Shalev, Stamberg & Simchen, 1972), *Stereum hirsutum* (Coates & Rayner, 1985b) and *Typhula incarnata* (Cavelier, 1982). Most of these examples probably involve both mitotic recombination and chromosome loss. In *Coprinus radiatus* recessive traits were frequently unmasked from heterozygous diploids indicating that chromosomal loss, without recombination might also occur (Prud'homme, 1970).

Partial diploids are often less viable than aneuploids or haploids (Käfer, 1960; Raper & Flexer, 1970). As already noted, this agrees with the low levels of PGM exhibited by *hirsutum* nuclei with rudimentary, as opposed to complete or transient genomes. The production of free-radical and reactive oxygen species which may initiate DNA repair mechanisms that lead to chromosomal loss has also been highlighted.

Haploid predominance

The persistence of haploidy throughout the life-cycles of the majority of the fungi, and its maintenance even in the heterokaryons of basidiomycetes (and some ascomycetes) contrasts with somatic nuclear conditions in most other eukaryotes. However, if we accept epigenetics as an essential aspect of fungal versatility and the concomitant dependence of this versatility upon the timing of germ-line sequestration, then the significance of haploid predominance in fungi becomes apparent.

In the genetic *milieu* that exists within a mycelium populated by haploid nuclei, genomes can undergo rapid independent epigenetic divergence, establishing many new forms that are open to selection, drift and 'mutation'. For an epigenetic system to become 'adaptive', a single genomic lineage must persist and undergo repeated cycles of selection. An early commitment to diploidy would break this continuity, constraining the ability of an individual genome to enter a novel

epigenetic state, and reducing the potential of the mycelium to respond adaptively. Furthermore, the establishment of a *tabula rasa*, or 'clean slate' following meiosis would remove any useful, or potentially useful epigenetic programs that had accumulated during the somatic phase.

Overall, diploidy reduces flexibility and for organisms with indeterminate life-forms, the costs may outweigh the benefits. The occurrence of diploidy in indeterminate organisms might therefore be viewed as the 'last-ditch' attempt to avoid conflict. A reliance on epigenetic sources of variation, late commitment to diploidy, late germ-line sequestration, and a preponderance of asexual modes of proliferation may all be linked.

Persistent haploidy also allows the alteration of nuclear ratios within heterokaryons, perhaps in an adaptive fashion (but note earlier discussions). The early formation of diploids would remove this possibility, imposing a strict 1:1 relationship. Since the maintenance of a haploid state also keeps open the possibility of escape should a conflicting relationship fail to resolve, it can be seen why this condition is key to the fungal life-style.

Nucleo-cytoplasmic interactions

In most eukaryotes cytoplasmic inheritance is maternal - why should this be the case? If we consider the interrelationships between nuclei and mitochondria then some relevant properties of nucleo-cytoplasmic interactions come to light.

Firstly, in forced interspecific mammalian heterokaryons, one, but not both, parents lose some of their chromosomes. (For example in mouse-human hybridomas it is the human chromosomes that are selectively eliminated). The parental genome that loses its chromosomes, also loses its mitochondrial DNA (De Francessco, Attardi & Croce, 1980). Moreover, increased viability of hybridomas can be achieved by poisoning the mitochondria of one species prior to fusion (Ziegler & Davidson, 1981).

Secondly, mammalian mitochondrial DNA is transcribed by special mtRNA polymerases which are encoded by the nuclear genome (Singer & Berg, 1990). The transcriptional start sites that these polymerases recognise differ from one mammalian species to another - accordingly the RNA polymerases are unique to each species. Logically therefore, mitochondrial DNAs can only co-exist with nuclei with the appropriate allelic variants of the mtRNA polymerase. Interspecific, and possibly even some intraspecific pairings may bring into contact nuclei and mitochondria that are therefore incompatible.

These observations both imply that the resident nucleo-cytoplasmic relationship in fungal mycelia is delicately balanced, and that the disruption of this balance could lead to untenable conflicts and degeneration (Rayner & Ross, 1988).

Upon release from a heterokaryon an invasive genome may be presented with a cytoplasmic partner with which it is not co-adapted. This could lead to the production of homokaryons with unstable phenotypes unless specific modifications occurred during a heterokaryotic association that promoted compatibility. Such a reliance upon modifications might also provides a rational basis for some of the patterns of mitochondrial inheritance and alteration observed in *Stereum*. Although more work needs to be done on the nature of the altered sequences, preliminary observations (Zac Watkins, personal communication) have indicated that the altered element contains a motif with homology to a number of linear mitochondrial plasmids.

Evolutionarily stable strategies and population divergence

Various ecological roles have been proposed for heterogenic incompatibility responses that engender a selective advantage. These include the restriction of outcrossing (Esser, 1971; Esser & Blaich, 1973); the limitation of exploitation of heterokaryons by nuclei with a proliferative advantage over their partners in a heterokaryon (Hartl, Dempster & Brown, 1975); protection against deleterious cytoplasmic factors (Caten, 1972), and even a role in the promotion of sexual development (Butcher, 1968; Labarére & Bernet, 1977). An alternative

proposition is that heterogenic incompatibility loci may not be adaptive at all, but merely a consequence of the association of disparate genetic information (cf Davis, 1966).

One of the potential consequences of conflict avoidance may be the sub-division of populations into partial, or complete intersterility groups. As a result of a co-evolutionary arms race between members of the population, suppressor / modifier systems of conflict attenuation might evolve which can restrict the range of partners that are compatible. When matings are established between allopatrically-derived strains, or where barriers to mating within a population are incomplete, the suppressors have no contextual relevance. This opens up both individual mycelia, and entire populations, to take-over (see earlier discussions). When sterility barriers are intact, populations may become divided internally into mixtures of isolated clones; each of which is capable of independent and therefore divergent evolution (Croft & Jinks, 1977).

Studies on the distribution of heterogenic incompatibility loci groups are commonplace as the starting point of population studies of plant pathogenic ascomycetes (see Glass & Kuldau, 1992; Leslie, 1993). In many plant pathogenic species, races or *formae speciales* are associated with specific hosts and are mirrored by their vegetative compatibility group (VCG) classification eg *Cochliobolus heterostrophus* (Leach & Yoder, 1983), *Cryphonectria parasitica* (Anagnostakis, 1977), *Erysiphe* (Wolf & McDermott, 1994), *Giberella fujikuroi* (Puhalla & Spieth, 1985) and *Verticillium dahliae* (Joaquim & Rowe, 1990). Sadly both the origins and significance of the incompatibilities remain obscure. Studies of *Fusarium oxysporum* have revealed the existence of VCGs which do not correlate with host or substratal preferences (Correll, 1991). *Fusarium*, and other organisms lacking a correlation between VCG and *formae speciales* might be good candidates for further studies on genomic conflict.

Some of the most revealing cases of population level conflict suppression arise from studies of fungi with pseudohomothallic breeding strategies. The nuclei within heterokaryotic mycelia of *Podospora anserina* and of other pseudohomothallic species such as *Neurospora tetrasperma*

or *Agaricus bisporus* have experienced long, and presumably harmonious co-evolutionary partnerships. The component genotypes within wild type isolates of *P.anserina* represent the association of the products of a common meiotic event which only separates the mating-type genes with a post-reduction frequency of about 98.9% Homokaryons derived from ascospores are therefore very rare in *P. anserina*, most of which arise from a failure of the post-reduction segregation mechanism, or to the aberrant partitioning of nuclei during spore formation (Raju, 1992). Similarly in *Neurospora tetrasperma* homokaryons are relatively rare (*ca* 10% of ascospores and 20% of conidia).

If the component nuclei of a pseudohomothallic species are separated, and then recombined with new partners, hidden incompatibilities are revealed. For example, when Jacobson (1994) separated the component genotypes from a heterokaryon of *Neurospora tetrasperma* and paired them against other homokaryons from different sources, deleterious combinations became apparent. The majority of the pairings (102/110), exhibited some form of sexual dysfunction. In rank order of severity the effects included complete sterility, female sterility, production of inviable or abnormal ascospores, the death of germlings following germination of ascospores, self-sterility and the production of progeny with only one mating-type. Furthermore, heterokaryons regenerated from homokaryons of allopatric origin were prone to breakdown even prior to spore formation.

The feedback that conflict can generate within a population becomes apparent when RFLP maps of homokaryons derived from a single source, from the same population, or different populations are compared (Merino, 1994). Homokaryons derived from a single source and the same population exhibited minimal variation. Markers were virtually isogenic at six linkage groups, and were only polymorphic at the linkage group supporting the mating-type locus. There appears to be very strong selection against genomic disparity in this organism.

Other studies on *Neurospora tetrasperma* have revealed some remarkable patterns of nuclear and mitochondrial exchange. Lee & Taylor (1993) reported on the replacement of one

population of mitochondria in a mycelium by another (cf *Stereum* work). Here the mitochondria from a resident 'maternal' acceptor mycelium replaced the mitochondria within a donor mycelium in those pairings that resulted in unilateral nuclear migration. This only occurred in allopatric matings, suggesting that their may be some population level differentiation of the loci responsible for controlling nucleo-cytoplasmic relations in this fungus and that mitochondrial populations possess different properties before and after mating.

Concluding remarks

Our overall understanding of the mechanisms responsible for homogenic and heterogenic incompatibility have been greatly enhanced by the implementation of molecular studies. However, as this thesis demonstrates, much still remains to be discovered, let alone explained. No doubt, with continued efforts, some of these areas will be uncovered in the chief organisms of cell biological and mycological research. It should not be forgotten though that the model organisms may be poor representatives of the fungal kingdom as a whole.

A bewildering range of mating behaviours are exhibited by this phylogenetically diverse group (see Ainsworth & Bisby's Dictionary of the Fungi, 1995 for a recent interpretation of this diversity). Many questions of a fundamental nature remain to be adequately broached. Why are sexually outcrossing ascomycetes predominantly dimictic, and the basidiomycetes diaphoromictic? In diaphoromictic species, how is mating-type diversity generated, and what is its true significance? What are the developmental and ecological consequences of bipolar and tetrapolar mating-systems? Why can basidiomycetes tolerate such a diversity of nuclear conditions? Indeed, why do fungi produce heterokaryons at all, when their occurrence amongst the remainder of the world biota is somewhat rare?

Answers to some of these questions will no doubt arise from further molecular studies of mating behaviour in fungi, but many will also require the integration of observations made at developmental, population biological, ecological and evolutionary scales of organization.

In conclusion, the players in the mating game, be they nuclei, mitochondria, chloroplasts or plasmids, are offered the promise of increased short-term versatility / adaptability, as well as longer-term viability and representation in future generations. By allowing other genetic entities access to their genetic territory, the players in this game also invite an alternative, more undesirable outcome, potentially leading to cellular degeneration and loss of individuality. The final outcome will depend largely upon the ability of the interacting parties to attenuate, or accommodate the incompatibilities that give rise to conflict.

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